CALLUS FORMATION

Symposia Biologica Hungarica 7

CALLUS FORMATION SYMPOSIUM ON THE BIOLOGY OF FRACTURE HEALING

Akadémiai Kiadó, Budapest

CALLUS FORMATION
SYMPOSIUM ON THE BIOLOGY OF FRACTURE HEALING

Edited by

ST. KROMPECHER and E. KERNER

(Symposia Biologica Hungarica 7)

The complex clinical problem of bone union is discussed, emphasizing the requirement for primary fractures healing. The structures of bone and cartilage are dealt with, as well as their connective and granulation tissues, which all participate in bone regeneration. The problems of the normal development, regeneration, transplantation and pathology of bone are discussed, based on extensive histogenetic, histochemical histopathological, electron-microscopic as well as biochemical investigations. A detailed description of the histochemical, biochemical and metabolic factors in callus formation is given, and the importance of vascularization of the callus is treated. In connection with the biochemistry of callus, certain problems of molecular biology are discussed. Through the above theoretical investigations, the aim of the Symposium was to improve the clinical treatment of fractures.
The volume contains 34 papers and 26 short reports which were presented at the Symposium as demonstrations.

AKADÉMIAI KIADÓ

PUBLISHING HOUSE OF THE
HUNGARIAN ACADEMY OF SCIENCES

Budapest

Symposia
Biologica
Hungarica
7

SYMPOSIA BIOLOGICA HUNGARICA

REDIGIT

ST. KROMPECHER ET E. KERNER

VOL. 7

AKADÉMIAI KIADÓ, BUDAPEST 1967

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TRANSLATIONS BY

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THE SYMPOSIUM WAS HELD AT DEBRECEN, 5–8 JULY, 1965

PRINTED IN HUNGARY

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Note: papers whose titles are marked with an asterisk were not read at the Symposium but were displayed and discussed in the demonstration room.

Symp. Biol. Hung., 7 pp. 1—3 (1967)

OPENING ADDRESS

by

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IT IS my pleasant duty to welcome you in Debrecen on the occasion of the Symposium on Callus biology, sponsored by the Department of Biology of the Hungarian Academy of Sciences. First of all, I wish to greet our guests who have come from abroad to participate in this Symposium. We highly appreciate that the best known and most active representatives of this special field have gathered here to give new perspectives to the development of our knowledge on callus formation, bone regeneration, etc.

I wish to greet Mr. M. Valkó representative of the local authorities; Dr. G. Szilágyi, President of the Municipal Council of Debrecen; Prof. P. Szarvas, Rector of the Kossuth Lajos University, Professor of Analytical Chemistry; Prof. P. Juhász, Rector of the Medical University, Professor of Neurology and Psychiatry, and all the professors and doctors of our University.

I should also like on behalf of the Minister of Health, Dr. Z. Szabó, and of the Biological Department of the Hungarian Academy of Sciences to convey their best wishes for the success of the Symposium.

It is common experience that large specialist congresses have their increasing limitations, whereas symposiums dealing, e.g. with one organ examining it from a certain viewpoint but discussing its many-sided aspects as well, are becoming more and more frequent.

The subject of this Symposium is the *examination of the healing process of fractured bone* according to the present many-sidedness of our knowledge.

On the occasion of the opening of the Callus Symposium, may I draw your attention to some topical problems of rehabilitation following bone fracture.

All over the world and in our old continent, we are witnesses to the rapid advance of industrialization. In spite of the development of equipment for the protection of workmen, the number of industrial and traffic accidents is growing steadily. Consequently, the problem of fracture healing, i.e. of callus formation is not only in the foreground of interest, but its timeliness is increasing year by year.

It is a biological rule that the broken bone begins to proliferate on and near the surface of the fracture leading sooner or later to a better or worse repair.

The purpose of this Symposium is to find out those methods by which a more rapid fracture healing can be achieved and by which the union of the broken ends occur in *adequate* position and by means of a callus of good quality. If our ambition is to examine and intervene in the course of the mechanism of fracture healing by *directing* it to achieve better results, it is inevitable for us to study the *biology of the newly formed bone in its fundamentals.*

It is the task of this Symposium to reveal the newest achievements of *molecular biology* of supporting tissues—especially bone and cartilage—and bring them into connection with *electron microscopical, histochemical and biochemical processes,* examining at the same time the alteration of local tissular metabolism during differentiation of granulation tissue to cartilage or to bone.

As it is known, fractures bring about the *rupture of the local vascular system.* Therefore it is of decisive importance whether the granulating tissue has an adequate *vascular supply.*

It is important to know whether the callus which has developed is continuously supplied with capillaries, whether the conditions necessary for the development of primary bone are ensured, otherwise the bone tissue will develop indirectly by an intermediary stage of preformative cartilage or connective tissue.

On studying the biology of tissues involved in callus formation, the importance of *capillarization* becomes more and more manifest, since *oxybiotic metabolism can take place only in tissues having a good vascular supply.* This interrelation is another evidence of the unity of *form and function* in the field of callus formation, too.

It seems reasonable to lay stress on the recognition that *form and function constitute an inseparable unity in life.* The alteration of one member of this unity brings about the alteration of the other member. This recognition has both *theoretical* and *practical* aspects. By recognizing this correlation between form and function we are able to induce the organism to produce the structure required by us, i.e. cartilage, a new joint or a bony union, even at a site where no cartilage, joint or bone had ever existed. It has been known for decades that by changing function we are able to change the quality of the callus. It is less known, however, that by stimulating or depressing vascularization, promoting or inhibiting the morphological development of the vascular system, we are able to influence *local metabolism* and consequently the *quality* of the developing callus in the earliest stage of its development.

When at this Symposium the biochemical, physiological, microscopical and primarily clinical aspects of the callus will be discussed, this view of regarding bone as a *living unity* cannot be neglected. Therefore, bone should be regarded as a unity, i.e. the *bone* itself, its *marrow* (whether haematopoietic red marrow, or yellow or "gelatinous" marrow), its *periosteum* with special regard to its stage of activity, and its *vascular* supply (whether its arterial supply and venous flow are ensured). It should also be considered whether the bone is surrounded by muscles and soft parts, to what extent the limb is active and what are the circulatory, hormonal and nervous systemic conditions of the patient.

By raising the question of the unity of form and function, the problem of clinical and postoperative treatment is necessarily raised.

While in the first half of this century the best treatment of fractures was considered to keep the fractured limb completely immobilized, special stress being laid on the advantages of rest, today, in the treatment of fractures the disadvantages of immobilization have become more and more manifest as giving rise to "inactivity atrophy", a phenomenon well known to anatomists, physiologists and clinicians. Our future aim in fracture treatment is, naturally, to avoid inactivity atrophy. Moreover, we have to find the theoretical principles of that treatment which would spare the site of fracture and yet would avoid inactivity atrophy and would lead to a rapid and sound repair. The role of the surgeon in deciding which treatment should be applied in a certain case of fracture is, of course, of great importance.

One of the tasks of this Symposium is to co-ordinate the multiple theoretical investigations with the best methods of clinical practice in such a way that after a careful critical selection the theoretically well-founded methods should be actually recommended for the best treatment of patients.

The participants of this Symposium belong to different countries from the Atlantic to the Pacific Ocean and from the Mediterranean to the Baltic. They come from different countries, but, have specialized in the same direction and they have a common desire and purpose: by developing our knowledge on the biology of man's skeletal system and bone regeneration, to co-operate with increased special knowledge and with one accord for the benefit of mankind.

In the light of this thought, I open the Symposium.

Symp. Biol. Hung., 7 pp. 5—34 (1967)

CHEMICAL COMPOSITION AND METABOLISM OF CARTILAGE AND BONE

by

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IN THIS report we intend to take a general view of the more significant data concerning the chemistry and biochemistry of preosseous cartilage and bone with special reference to contributions of our school.

Embryologically several types of ossification can be distinguished. Biochemically it seems that the chemical factors responsible for the ossification process are very similar. The analytical data available in the literature are frequently discordant. This could be explained by considering the variability of the samples of analysed material and also the fact that ossifiable cartilage and bone are dynamic systems where the process of destruction and reconstruction take place actively in permanent evolution.

GENERAL COMPOSITION OF CARTILAGE AND BONE

INORGANIC COMPONENTS

We begin by considering the inorganic constituents of these tissues. The epiphyseal cartilage is a tissue very rich in water. According to the investigations of De Bernard and Castellani (1955) obtained in our laboratory, the percentage calculated for dried material at 110 °C may reach 77 to 78%. Table I compares this value with those of different types of cartilage.

TABLE I

Water in cartilage

Type of cartilage	%	Reference
Patella (human infant)	80·0	Eastoe (1961)
Costal (human infant)	85·0	Eastoe (1961)
Costal (human adult)	60·0	Eastoe (1961)
Costal (young dog)	74·0	Eastoe (1961)
Articular (young dog)	76·4	Eastoe (1961)
Nasal septum (ox)	76·9	Eastoe (1961)
Tracheal (ox)	66·6	Eastoe (1961)
Epiphyseal (rabbit 12—15 days)	77·6	De Bernard and Castellani (1955)
Epiphyseal (rabbit 30—40 days)	76·4	De Bernard and Castellani (1955)

These are average values which, however, can vary widely according to the cartilage type, age and animal species. It may be of interest that, according to Miles and Eichelberger (1964), the water content of human articular cartilage is constant until the age of 50, after which the extracellular water decreases, whereas the endocellular water in the chondrocytes remains constant.

The main inorganic constituents of cartilage are given in Table II.

TABLE II

Inorganic constituents of cartilage. The values are expressed in mg per 100 g of dry weight (after Eichelberger et al. 1951)

	Ox		Dog	
	Tracheal	Nasal	Costal	Articular
Cl^-	420	800	640	890
Na^+	2170	1980	1850	2200
K^+	340	757	780	980
SO_4^{--}	1470	1020	1000	1100
Ca^{++}	243	155	180	220
Mg^{++}	85	60	84	96

Note: the following elements were found in traces: Si (9 to 18 mg%; King and Belt 1938), and F (0·3 to 1·5 mg; De Eds 1963).

The bone also shows great variation in water content: this is a function of the type of bone, the animal species, age and anatomical portion. For human bone at different ages, data obtained by Dickerson (1962) are shown in Table III.

TABLE III

Water in fresh human femur (whole bone; after Dickerson 1962)

Age	%
Foetus 12—14 weeks	77·8
Foetus 30—40 weeks	63·4
New-born full term	63·8
Infant 1—2 years	52·1
Infant 11—12 years	27·0
Adults 18—35 years	15·6

The chemical composition of the mineral fractions of bone is summarized in Table IV. However, many other ions exist besides those previously listed, which are given in Table V.

TABLE IV

Chemical composition of mineral fractions of bone
(Zambotti 1960)

	%	Reference
Tricalcium phosphate	74·40	Cartier
Calcium carbonate	10·30	Cartier
Calcium citrate	2·00	Cartier
Calcium lactate	6·19	Cartier
Magnesium (Mg^{++})	0·50	
Potassium (K^{+})	0·30	Holtz and Schütte (1954)
Sodium (Na^{+})	0·80	Holtz and Schütte (1954)
Chloride (Cl^{-})	0·00	Holtz and Schütte (1954)
PO_4^{---} } bound to Ca^{++} } organic matrix	8·76	Cartier
H_2O (crystallization water)	2·50	Holtz and Schütte (1954)
Total:	99·75	
Pyrophosphate (referred to total P)	0·3—1·0	Perkins et al.
Ratio $\frac{\text{P (as pyrophosphate)}}{\text{P total}}$ =	0·47	Cartier

TABLE V

Inorganic constituents of bone (% of dried bone; after Eastoe 1961)

Ca		25·6
P		12·3
Mg		0·39
CO_2		4·0
Cl^{-}	0·17	— 0·19
F^{-}	0·061	— 0·31
Na	0·18	— 0·6
K	0·05	— 0·3
Fe	0·011	— 0·017
Cu	0·0002	— 0·0048
Pb	0·001	— 0·01
Mn	0·00005	— 0·0022
Al	0·00024	— 0.0005
Sr	0·00001	— 0·0002
B	0·0016	— 0·014

Note: spectrographically the following have been detected: Ag, As, Ba, Bi, Li, Mo, Ni, Se, Si and Zn.

ORGANIC COMPONENTS

More than 80% of the dry weight of cartilage is composed of organic substances (including cellular material). The principal ones are represented by collagen, non-collagen proteins, mucoproteins and mucopolysaccharides. Lipids, glycogen, organic acids, nucleic acids, enzymes and coenzymes,

amino sugars, uronic acid are present in smaller quantities, but are, nevertheless, of great biochemical significance.

Organic acids. The presence of pyruvic, lactic, α-ketoglutaric, citric, aconitic, succinic and malic acids have been determined by chemical analysis. Perri et al. (1955) demonstrated the presence of pyruvic and ketoglutaric acids in rabbit epiphyseal cartilage in our laboratory. They found 4·05 mg of pyruvic acid and 1·46 mg of ketoglutaric acid per 100 g of fresh tissue. In the epiphyseal cartilage of the rabbit (Fiandre strain, 15 to 20 days old), the concentration of pyruvic acid is higher (6·1 mg per 100 g of fresh tissue), but it decreases with age: it was 3·3 mg per 100 g at 60 to 65 days of age. The lactic acid content in sheep epiphyseal cartilage is 0·6 to 3·8% (Eastoe 1961).

Dickens (1941) showed that over 90% of the body citrate is present in the skeleton. The most important data concerning the distribution of citrate in the bone are given in Table VI.

TABLE VI

Citrate in bone

Species	Values (mg/g)	Reference
Human (bone)	8·9	Eastoe (1961)
Ox (bone)	4·75	Lees and Kuyper (1957)
Dog (bone)**	9·09 — 5·53	Lees and Kuyper (1957)
Dog (bone)*	8·52 — 9·66	Lees and Kuyper (1957)
Rabbit (bone)**	3·94 — 5·09	Lees and Kuyper (1957)
Rabbit (bone)*	5·65 — 6·27	Lees and Kuyper (1957)
Rabbit (bone)	8·3 — 0·13	Cartier (1957)
Rat (metaphysis)	6·7	Dixon and Perkins (1952)
Rat (epiphyseal line)	7·1	Dixon and Perkins (1952)
Rat (bone cortex)	9·5	Dixon and Perkins (1952)
Cod	20·1	
Herring	50·0	Thunberg (1948)

Note: * = cancellous bone
** = compact bone.

In Table VII the citric acid concentration is compared with that of aconitic, succinic, fumaric and malic acids.

Lipids. The presence of lipids and phospholipids in cartilage was demonstrated by Borghese (1936). Irving (1965) confirmed this finding in ossifying tissues.

In 1960 Tinacci and Cioni demonstrated with histochemical technique the presence of triglycerides, cholesterol and phospholipids in the epiphyseal cartilage. Zambotti et al. (1962) demonstrated the presence of lipids in epiphyseal cartilage of new-born pigs, and also determined the non-esterified and the esterified fatty acids of the same tissue by gas-chromatographic procedures. Cescon and Zambotti (1962) also reported the analysis on blood

TABLE VII

Organic acids in bone (after Lees and Kuyper 1957)

Tissue	Organic acids (mg per 100 g)				
	Citric	Aconitic	Succinic	Fumaric	Malic
Bone ox*	475	2·7	1·8	0·2	5·2
Bone dog A*	852	3·6	2·7	0·2	6·6
Bone dog A**	609	3·3	3·0	0·2	3·9
Bone dog B*	966	3·8	1·7	0·1	5·0
Bone dog B**	553	1·6		0·2	4·9
Bone rabbit A*	565	2·6	3·6	0·2	6·3
Bone rabbit A**	394	2·2	3·1	0·2	5·3
Bone rabbit B*	627	3·6	1·3	0·2	4·6
Bone rabbit B**	509	2·0	2·6	0·2	8·0
Egg-shell A	66	0·0	0·0	0·0	2·9
Egg-shell B	11	0·0	0·0	0·0	0·8
Rat liver	3·3	0·1	2·5	0·5	1·6

Note: * = cancellous bone
** = compact bone.

and on nasal septa of the same animals for comparison with the epiphyseal cartilage. The results are presented in Tables VIII and IX.*

TABLE VIII

Lipids in cartilage and blood of new-born pigs
(after Zambotti et al. 1962)

	Cartilage		Blood (%)
	Epiphyseal (%)	Nasal (%)	
Total lipids	6·60	0·68	4·1
Total phosphorus (as P)	3·43	0·44	0·23
Lipidic phosphorus (as P)	0·054	0·013	0·067
P total / P lipidic	63·50	34·30	4·10

The tables furnish evidence that the total lipid content in the epiphyseal cartilage of new-born pigs is higher than in the blood (6·6% as against 4·1%), and about ten times higher than in the nasal septum.

As regards fatty acids it is worth while to point out the large content of poly-unsaturated acids of the epiphyseal cartilage compared with that of the nasal septum. According to Wuthier (1965) reported by Irving (1965), the hypertrophic cell layer has three times as much total lipid as the

* The lipids and phospholipids in rat fracture callus of 10 days and in the blood of the same animals have been extracted and determined by Cescon and Bolognani (1962); the same determinations have also been repeated in the blood of the controls. No significant differences have been found between the last values.

TABLE IX

Fatty acids in cartilage

Acids	Cartilage		Blood (%)
	Epiphyseal (%)	Nasal (%)	
Saturated	38·50	43·40	38·70
Mono-unsaturated	44·30	40·20	47·60
Poly-unsaturated	17·20	15·10	13·70
Lauric	0·20	1·30	0·10
Y-tetradecanoic	0·10	0·10	0·50
Myristic	2·30	2·50	1·20
Myristoleic	0·40	0·40	0·10
N-pentadecanoic	1·00	1·10	0·40
R-hexadecanoic	0·90	0·30	0·10
Palmitic	19·90	25·40	24·90
Palmitoleic	8·20	7·40	6·20
N-heptadecanoic	0·60	1·10	1·00
Heptadecanoic	1·10	1·20	0·90
Stearic	13·40	11·00	10·50
Oleic	34·10	30·20	40·20
Linoleic	4·30	1·40	7·20
Linolenic	0·60	2·10	0·10
Arachidic	0·10	1·00	0·20
Eicosenoic	0·50	0·10	0·10
Eicosadienoic	1·40	1·60	0·10
Eicosatrienoic	2·10	1·60	0·40
Arachidonic	6·50	4·00	3·10
Others	2·30	5·20	2·70
Total:	100·00	99·00	100·00

neighbouring layers. It is also important to mention the presence in the epiphyseal cartilage of considerable amounts of polar lipids, lecithins, phosphatidyl serine, phosphatidyl ethanolamine and phosphatidyl inositol. Sphyngomyline, phosphatidic acids and an acid component not yet clearly identified are also present, although in smaller amounts.* The unidentified acid component can increase considerably, especially in the hypertrophic cell layer, and so it becomes the predominant lipid (Wuthier 1965).

The bone tissue is rather poor in lipids: the compact bovine femur contains lipids at a concentration of only 0·7 to 0·1% of the dry weight (Spector 1956). The main lipid fractions of compact bone are given in Table X.

Nucleotides. A number of nucleotides have been found both in cartilage and bone. The available data only concern cartilage, and they are summarized in Table XI.

* The acid component does not contain ions as PO_4^{---} and SO_4^{--} nor free amino groups or very unsaturated bonds.

TABLE X

Composition of lipids from compact bone (ox femur diaphysis) after Leach 1958

	g/100 g of total lipid	g/100 g of dry bone
Triglycerides	79·2	0·0535
Free cholesterol	13·3	0·0089
Cholesterol esters	1·7	0·0011
Phospholipids	2·2	0·0015

TABLE XI

Nucleotides in epiphyseal cartilage

ATP	Albaum, Hirschfeld and Sobel (1952)
ADP	Cartier and Picard (1955)
AMP	Tancredi, Ipata and Musii (1958)
GMP	De Bernard, Bianco and Zambotti (1959)
GDP	De Bernard, Bianco and Zambotti (1959)
GTP	De Bernard, Bianco and Zambotti (1959)
CMP	De Bernard, Bianco and Zambotti (1959)
CTP	De Bernard, Bianco and Zambotti (1959)
UTP	Bianco, Castellani, De Bernard and Zambotti (1958)
UMP	Bianco, Castellani, De Bernard and Zambotti (1958)
UDP glucose	Bianco, Castellani, De Bernard and Zambotti (1958)
UDP Ac. glucosamine	Bianco, Castellani, De Bernard and Zambotti (1958)
UDP Ac. gal. sulphated	Picard, Gardais and Dubernard (1964)
PAPS	Picard, Gardais and Dubernard (1964)
Inosin necleotides	Tancredi, Ipata and Musii (1958)

It is likely that nucleotides are also present in bone, in osteocytes and osteoblasts; unfortunately, we have no information, probably because their determination is technically quite difficult.

Nucleic acids (RNA and DNA). The presence of nucleic acids both in cartilage and bone cells has been established mainly by histochemical procedures. Small amounts of RNA are reported in the resting cartilage cells, whereas it is present in higher concentrations in proliferating and hypertrophic cells. In the cartilagineous cells DNA is also present as demonstrated by Pritchard (1952) and Follis (1952); Gerzeli and Bottino (1957) confirmed this finding by using histospectrophotometric techniques. The DNA concentration in the epiphyseal cartilage of new-born pigs ranges between 1·19 and 1·32 mg per 100 mg of dry weight, whereas in young rabbits it is 0·83 to 1·13 mg* (Kofler 1965). RNA and DNA have been determined in bone, too; RNA content is high in osteoblasts (Bhaskar et al. 1956); it increases until the bone mucoproteins are laid down, but decreases when osteoblasts

* DNA in the fracture callus of rats changes from 1·25 mg per 100 mg of dry weight at five days to 0·97 mg at 20 days after fracture (Bolognani and Marcheselli, unpublished data).

are converted into osteocytes (Cappellin 1948, 1949). Pritchard (1952) and Follis (1952) demonstrated and assessed DNA in the nuclei of preosteoblasts, osteoblasts and osteoclasts.

Collagen. As is well known, collagen is an insoluble protein which has been exhaustively investigated (Eastoe and Leach 1958, Watson and Silvester 1959, Gross and Piez 1960, Gross 1963, Orekhovich and Shpikiter 1958, Banga and Baló 1960).

Collagen is largely present in cartilage and bone. In cartilage the following are reported: dry ox trachea contains 43% of collagen, nasal septum 47%, dog costal cartilage 44% and articular cartilage 39%.

In the bone the collagen (expressed as a percentage of dry defatted bone) decreases with age from 25 to 22·3%, but it is interesting that over 90% of bone organic matter consists of collagen (Eastoe 1955).

Collagen is also formed in these tissues from tropocollagen. It is likely that tropocollagen has terminal parts sensitive to proteolytic enzymes (Nishihara et al. 1963). On studying the effect of proteolytic enzymes on soluble and insoluble collagen, it has been found that terminal parts are poor in hydroxyproline and rich in tyrosine, aspartic and glutamic acids which are hydrolysed by protease and are very likely involved in polymerization processes (Rubin et al. 1963, Gallop 1964).

Since it may concern mineralization, prominent interest has been attributed to the ε-amino groups of lysine and hydroxylysine which are present in collagen as will be discussed later. According to Wuthier et al. (1964), the ε-amino groups should be classified in four groups, depending on their probable functional meaning:

Group 1 is bound with electrostatic bonds to hydroxyapatite (concerning 25 to 30% of total number of free ε-amino groups);

Group 2 seems to be more firmly bound to the mineral fraction (concerning 25% of total ε-amino groups), so that they do not react with flourodinitrobenzene (FDNB) unless previous demineralization of bone tissue has taken place;

Group 3 would be involved in the covalent cross-linkages of collagen (including 21% of ε-amino groups);

Group 4 does not bind mineral matter (including about 25% of ε-amino groups).

Chondroitin sulphate and mucoproteins. The presence of mucopolysaccharides (MPS) in the cartilage has been known for many years. Krukemberg (1884), quoted by Schubert (1964), first extracted them with NaOH. Mörner (1889), Blix and Snellman (1955), Einbinder and Schubert (1950) and many others followed Krukemberg.

There are, indeed, different types of chondroitin sulphates; their repeating unit is given in Fig. 1.

Only CSA, CSC and KS have been isolated in the cartilage and bone, while CSB seems to be lacking. The distribution of these substances is shown in Table XII.

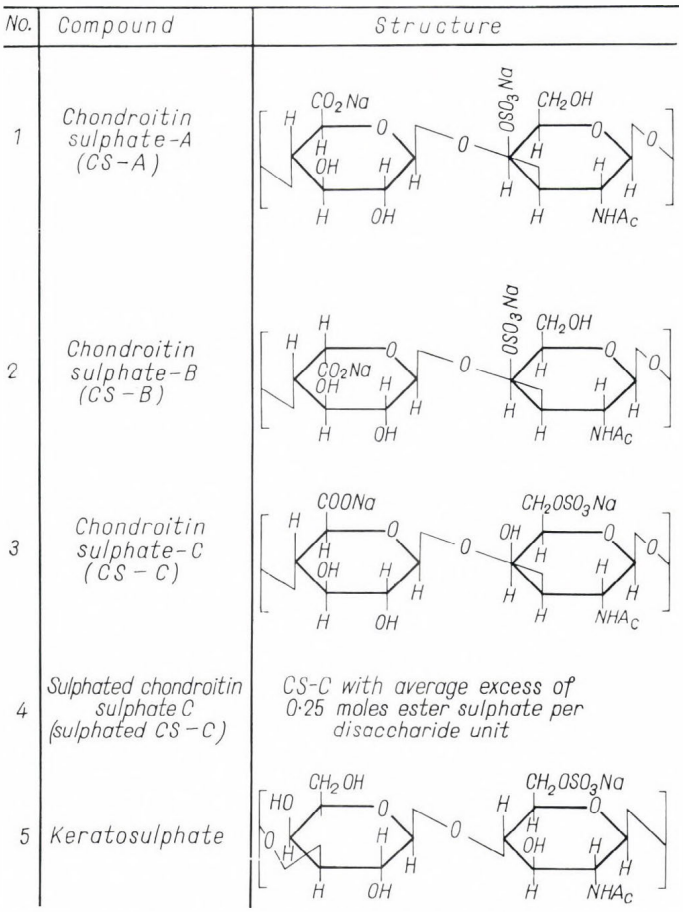


FIG. 1. Polymer disaccharide units (after Mathews 1964)

Protein-polysaccharide complexes (PP). It has been postulated long ago that CS could combine with some protein-forming mucoproteins. Shatton and Schubert demonstrated this in 1954 and later Muir (1956) and Partridge and Davis (1958) confirmed it. Two of these mucoproteins have been separated by ultracentrifugation (one at 50,000 g and the other at over 100,000 g) and characterized chemically.

These complexes are designated as PPL (protein polysaccharides, light) and PPH (protein polysaccharides, heavy). PPL contains about 85% of CS and 15% of protein. PPH is much richer in proteins. The analytical data of PPL and PPH are given in Table XIII.

These protein polysaccharides have been found in nasal cartilage (Gerber et al. 1960, Gregory et al. 1961, Scheinthal and Schubert 1963), in the epiphyseal cartilage of new-born pigs and in human cartilage (Castellani et al. 1961), and in costal cartilage (Campo and Dziewiatkowski 1962).

TABLE XII

MPS distribution in cartilage

Cartilage	Total	CSA	CSC	KS	Ch	Reference
Ox tracheal	19·8	34·9	42·4	22·7		Greiling et al. (1964)
Human articular						
young	6·4	58·2		39·3	2·9	Greiling (1964)
old	6·8	44·2		53·0	2·8	Greiling (1964)
Human costal						
young			12%	1%		Kaplan and
old			1%	45%		Meyer (1959)
Rana catesbiana						
embryo		40	20		40	Mathews and
embryo developed		50	50			Hinds (1963)
after metamorphosis		70	30			
Calf scapula						
cartilage	24·0	+	+			Lindenbaum
bone	6·5	+	+			(1963)
Pig epiphyseal						
ETOH 40%		60%	40%	+		Castellani et al. (1962)
ETOH 50%			+	++		

TABLE XIII

Analytical data on PPL and PPH

Material Bovine	Complex	Peptides	Uronic	Hexosamines	Hexose	Sialic	S	Reference
Nasal cartilage	PPL	15·1	25·1	26·7 (gal)			4·6	Gerber (1960)
	PPH	56·5		19·6			3·0	
Nasal cartilage			22·6	2·5 (glu)	7·0	2·2	4·7	Gregory et
				21·8 (gal)				al. (1961)
Costal cartilage				2·2 (glu)				
	PP	25·2	21·7	21·9 (gal)			3·02	Campo et
	PPL	17·8	25·6	1·5 (glu)			3·83	al. (1962)
				25·9 (gal)				
	PPH	67·9	10·2	2·1 (glu)			2·34	
				9·7 (gal)				
Metaphyseal cartilage pig	PPL	6·5	26·9	1·31 (glu)	2·02	0·54	4·59	Castellani et al. (1962)
				23·87 (gal)				
Nasal cartilage	PPL	15·3	25·1	2·5 (glu)	5·9	0·80		Scheinthal et al. (1963)
	PPH			24·1 (gal)				
	PPH	53·0		2·9 (glu)	5·0	0·53		
				16·6 (gal)				
Nasal cartilage	PPL		24·9	2·1 (glu)	7·3	2·2	4·7	Gregory et
				18·1 (gal)		0·84		al. (1964)
Nasal cartilage	PPL	18·9	23·0	25·4	5·1 (2·5)	0·8		Doganges and Schubert (1964)

As regards the nature of the chemical bond between protein and polysaccharides, Muir proposed in 1958 that it is contributed by serine. The work of Castellani et al. (1962), Anderson (1964) and Gregory et al. (1964) proved this. The protein—carbohydrate linkage has the following general structure (Gregory et al. 1964):

The bond between CS and sugar is of glucuronidic type, and the one between galactose or xylose and serine is glycosidic.

It should be pointed out that PPL and PPH do not contain hydroxyproline, and this justifies the denomination of "non-collagenous proteins."

The bone also contains complexes between proteins and polysaccharides as shown by Hawk and Gies (1901), quoted by Eastoe (1956). These authors isolated a complex named "osseomucoid" which was fractionated by Hishamura (1938), quoted by Herring and Kent (1963). Relevant studies on the extraction and purification of bone mucopolysaccharides are those of Eastoe (1956), Glegg and Eidinger (1955), Meyer (1956), Dische et al. (1958) and King and Boyce (1959).

Sialic acid and sialoproteins. A new contribution to the biochemistry of metaphyseal cartilage was reported from our laboratory by Castellani et al. (1959, 1960) and Bolognani and Laneri (1961) who demonstrated the presence of considerable amount of sialic acid (Table XIV).

Anderson (1961, 1962) confirmed the presence of sialic acid. He also demonstrated that sialic acid in the glycoproteins of human cartilage is located in a terminal position.

Sialoproteins are present also in bone tissue as demonstrated by Herring and Kent (1963). The sialoprotein extracted by these authors contained

TABLE XIV

Sialic acid in cartilage (values are expressed as μg/g of wet tissue)

Epiphyseal cartilage		Costal cartilage
New-born pigs	Young rabbits	Young rabbits
1651	707	620
	681	
1355	968	579
	941	
1900	867	487
1586	940	705
	867	
	745	
	1170	
Av. 1618	Av. 876	Av. 597

about 17% of sialic acid and is quite different in composition from orosomucoid. The differences are clearly shown in Table XV.

TABLE XV

Bovine bone sialoprotein and serum orosomucoid
(after Herring 1964)

	Sialoprotein (%)	Orosomucoid (%)
Nitrogen	10·4	11·2
Hexose	10·3	13·3
Methylpentose	2·3	0·8
Glucosamine	3·9	7·2
Galactosamine	3·8	0·7
Sialic acid	17·1	11·5
Phosphate	1·8	0·2

THE METABOLISM OF CARTILAGE

General metabolic pathways described for other tissues have been demonstrated in cartilage where glycolysis, tricarboxylic acid cycle and hexose-monophosphate shunt have been observed.

Glycolysis is particularly important in preosseous cartilage. Great importance is ascribed to this process in explaining the origin of organic phosphates which are necessary for calcification (Creighton 1896, Hoffmann et al. 1928, Harris 1932, Glock 1940, Gutman and Gutman 1941, Cobb 1953). The chemical pathways of glycolysis are proved by the presence of the enzymes which catalyse this metabolic pathway, and by related metabolic intermediates. The enzymes dealing with glycolysis are summarized in Table XVI.

TABLE XVI

Enzymes in glycolysis

	In cartilage	In bone	Reference
Glycogen phosphorylase	+		Gutman et al. (1941)
	+		Cobb (1953)
Phosphoglucomutase		+	Cabrini (1961)
Hexokinase	+		Gutman et al. (1950)
Phosphohexose isomerase	+		Albaum et al. (1952)
Phosphofructose kinase	+		Albaum et al. (1952)
Aldolase	+		Albaum et al. (1952)
Triosephosphate isomerase	+		
Triosephosphate dehydrogenase (DPN)	+		Albaum et al. (1952)
Phosphoglycerate kinase	+		Delbrück (1964)
Phosphoglycero mutase			
Enolase	+		Albaum et al. (1952)
Pyruvate kinase	+		Delbrück (1964)
Lactic acid dehydrogenase	+		Albaum et al. (1952)
	+		Delbrück (1964)
		+	Balogh et al. (1961)

Phosphorylase is present in every part of the limb cartilage (Grillo 1964). Delbrück (1964) found a considerable amount of 3-phosphoglyceraldehyde dehydrogenase; lactic dehydrogenase and phosphoglycerylkinase are also very active. On the other hand, the enzyme catalysing glycogen synthesis from UDPG has been demonstrated in the middle part of the limb cartilage of an 8-day-old embryo (it seems to be limited to this part, Grillo 1964).

Hexosemonophosphate shunt. It is well known that the metabolism of glucides may follow another very interesting way, the HMS (hexosemonophosphate shunt). As regards the HMS, the first experimental data to support the occurrence of this cycle in epiphyseal cartilage were obtained in our laboratory in 1958 by Bolognani and Ferri who demonstrated transketolase activity in metaphyseal and costal cartilage (Table XVII).

TABLE XVII

Transketolase activity in cartilage (the values are expressed in μg/ml)

	Pentose at time 0	Pentose disappeared after		Heptulose synthesized after	
		15 min	60 min	15 min	60 min
Metaphyseal cartilage	50	4	14	0·6	1·9
	46	10	26	0·7	2·3
	46	5	21	1·2	2·4
	46	6	15	0·8	2·5
	Average	6·1	15	0·8	2·2
Costal cartilage	50	3	5	0·9	1·3
	46	4	12	0·6	0·9
	47	2	5	0·4	1·2
	46	5	9	0·2	1·2
	Average	3·5	7·9	0·5	1·1

The G—6—P—dehydrogenase demonstrated by Kuhlman (1960) also exists.

The hexosemonophosphate shunt is a very important process; it produces, *inter alia*, the phosphorylated pentoses for the biosynthesis of nucleotides and of nucleic acids; it is also an important source of energy and it produces NADPH indispensable for many biosynthetic reactions.

KREBS CYCLE

The presence of the Krebs cycle in epiphyseal cartilage has been supported by the presence of the proper enzymes, coenzymes and related metabolites. The enzymes whose presence has been ascertained are given in Table XVIII.

Among the coenzymes those of oxidative decarboxylation have been well described (cocarboxylase, NAD,$^+$ FAD).

TABLE XVIII

Enzymes of tricarboxylic acid cycle and respiratory chain

	In cartilage	In bone	Reference
Citrogenase	+	+	Dixon et al. (1952)
	+	+	Norman et al. (1964)
Aconitase	+	+	Dixon et al. (1952)
		+	Hekkelman (1963)
Isocitric dehydrogenase	+	+	Dixon et al. (1952)
	+		Delbrück (1964)
		+	Van Reen et al. (1958)
		+	Hekkelman (1963)
	+		Follis et al. (1956)
Succinic-dehydrogenase	+		Follis et al. (1956)
	+		Castellani et al. (1954)
	+		Follis et al. (1956)
	+	+	Balogh et al. (1961)
			Shajowicz et al. (1960)
Fumarase	+		Kuhlman (1960)
Malic dehydrogenase	+		Follis et al. (1956)
	+		Delbrück (1964)
DPNH cyt. C-reductase	+	+	Balogh et al. (1961)
	+		Fine et al. (1963)
		+	Hekkelman (1963)
TPNH cyt. C-reductase	+	+	Balogh et al. (1961)
		+	Hekkelman (1963)
Succinate cyt. C-reductase		+	Hekkelman (1963)
Pyridin nucleotide transhydrogenase		+	Hekkelman (1963)
Cytochrome C			Lutwak-Mann (1940)
Cyt. C-oxidase	+		Fine et al. (1963)
		+	Cabrini (1961)

The presence of cocarboxylase in the epiphyseal cartilage of young rabbits was demonstrated by Zambotti and Lorenzi in 1953. Cocarboxylase concentration decreases with the animal's age and when Vitamin B_1 is lacking in the diet as observed in our laboratory by De Bernard and Lorenzi (1955).

The presence of succino-codehydrogenase, observed by Follis and Melanotte (1956), has subsequently not only been confirmed in our laboratory with colorimetric techniques, but we were able to demonstrate a stronger activity in the epiphyseal cartilage than in the costal cartilage (Castellani and Bianco 1956), and higher concentration in the hypertrophic cell layer of this type of cartilage (Castellani and Zambotti 1954). Balogh et al. (1961) confirmed this finding.

During development, malic dehydrogenase decreases sharply in articular cartilage, while isocitric dehydrogenase practically does not (Delbrück 1964).

It has been suggested that isocitric dehydrogenase may explain why citrate concentration reaches such high values in the growing sites of ossifying cartilage.

The first explanation considered the lack of isocitric dehydrogenase as the major responsible factor. However, this hypothesis was discarded when

Van Reen (1959) demonstrated that this enzyme does exist in a considerable amount in the bone.

Today we believe that citric acid increase must be due not to the lack of isocitric dehydrogenase, but to a lack of the proper coenzyme: NADP.

It is well known that the Krebs cycle occurs under aerobic conditions and it is strictly dependent on respiratory processes. The respiratory activity of the cartilage has been demonstrated with manometric and spectrophotometric techniques. The respiratory activity of the cartilage varies according to embryonal development and differs from one cartilage type to another. Boyd and Neuman (1954) found QO_2 ranging about 2·2 in 14-day-old chicken embryo cartilage; such values decrease by 50% in 20-day-old embryos and are 1/200 of the embryonal values in adult animals.

Oxygen consumption is catalysed through the cytochrome-oxidase system which is present in cartilage. Lutwak-Mann (1940) demonstrated cytochrome-C in cartilage, and Boyd and Neuman (1954) observed cytochromoxidase activity. Fine and Pearson (1963), using spectrophotometric techniques succeeded in proving a five times higher cytochromoxidase activity in the epiphyseal cartilage than in the xiphosternal samples.*

In conclusion, we may observe the Krebs cycle in the ossifiable cartilage where it finds particularly favourable conditions at the site of the hypertrophic cell layer, largely by the oxygen supplied.

The main functions of the Krebs cycle are strictly related to the production of several important metabolites, but first of all to furnish large amounts of ATP indispensable for bone salt formation. The observation (Cartier 1952) that ATP concentration is very high, particularly at the site of the mineralization line where values comparable with those of the liver have been found, is in agreement with this statement.

Metabolism of MPS in cartilage. A particularly active and important metabolic process which is also the subject of extensive research today is the biosynthesis of MPS and precursors (Table XIX; Zambotti 1961).

Distant precursors for the biosynthesis of MPS are: glucose as source of carbon skeleton, glutamine as donor of amino groups, and PAPS.

Fundamental intermediates are the acetylated and activated N-acetylhexosamines (UDPN-ac. hexosamines), the active uronic acid (UDP glucuronic acid) and its precursor: the UDPG. It is commonly believed that UDPG is synthetized by interaction between G—1—P and UTP, but researches of Tettamanti (1961) and Tettamanti and Bertona (1962) on the epiphyseal cartilage suggest that this intermediate could be synthesized also directly by a transglycosidation mechanism from glycogen, following the reaction:

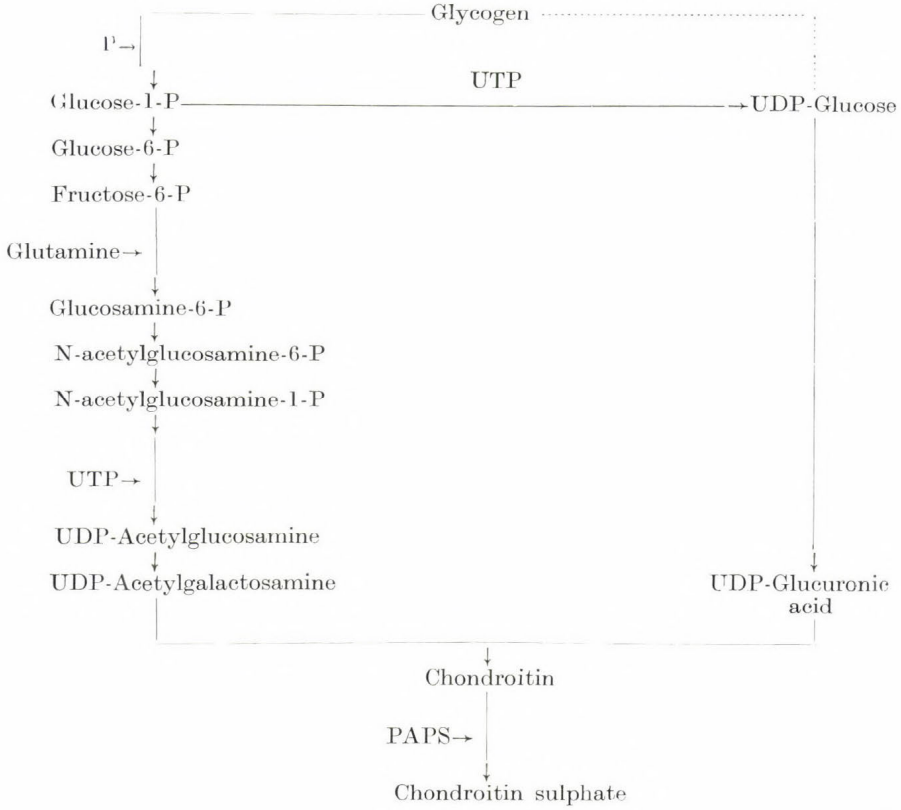
$$(\text{Glucose})n + \text{UDP} \dashrightarrow (\text{Glucose})n - 1 + \text{UDPG}$$

This process, if confirmed, could be very interesting since it may be a source of active glucose without any ATP or UTP consumption, but using the energy of the glycosydic linkage in the glycogen molecules only.

* Probably the cytochrome-cytochromoxidase activity exists in the bone callus' too, as Földes et al. (1964) demonstrated a considerable respiratory acitivity in fracture sites.

TABLE XIX

Biosynthesis of chondroitin sulphate



Distant precursors as glucosamine—6—P and galactosamine—6—P are also synthesized from the epiphyseal cartilage starting from glucose—6—P and glutamine. The reaction described first by Leloir and Cardini (1953) in *Neurospora crassa*, was demonstrated in homogenates of epiphyseal cartilage (Castellani et al. 1955). Thereafter, hexosamine becomes acetylated (Boström and Manson 1952).

An extensively discussed problem concerns the sulphurylation process; for interesting contributions we are indebted to Dziewiatkowski (1951), Boström (1952), D'Abramo et al. (1958), Amprino (1958) and Adams (1963).

The more controversial matter is whether the sulphate is incorporated before or after polymerization of the heteropolysaccharides has taken place. Actually, the second seems to be more acceptable. Recently Pearlman et al. (1964) demonstrated that the cell-free enzyme preparation catalyses the synthesis of non-sulphated chondroitin which is subsequently sulphated. The biosynthesis of MPS is stimulated by several factors, of these we men-

tion glutamine and nucleotides as ATP and UTP. Namely, glutamine stimulates incorporation of SO_4^{--} in the MPS (Boström 1952) and homogenates of epiphyseal cartilage in the presence of ATP, and glutamine and $MgCl_2$ incorporate SO_4^{--} in highly polymerized material (Castellani et al. 1962).

Another aspect of the problem concerns the simultaneity of the biosynthesis with the protein which will be bound with MPS in order to build up a protein-mucopolysaccharide complex.

According to Campo and Dziewiatkowski (1962), the polysaccharide and protein are synthesized simultaneously. By autoradiographic techniques it has been observed that the incorporation takes place mainly in chondrocytes.

Several other factors, which for lack of time cannot be discussed, may affect the biosynthesis of MPS, e.g. neural, hormonal and dietetic factors. It should be remembered that a low Cu diet may induce an increase of MPS in the cartilage compared with the controls (Linker et al. 1964).

Nucleic acid and metabolism. The cartilaginous cells are certainly able to synthesize nucleic acids. Tonna and Cronkite (1964) and Owen (1964) proved this with labelled precursors (tritiated tymidine).

The epiphyseal cartilage and the cells of the osteogenic periostal layer incorporated the tracer quite rapidly, but it only remained fixed for one month; the articular cartilage cells and those of the periosteal fibrous layer incorporate a smaller amount of the tracer, but it remains fixed longer.

Biosynthesis of proteins. The cartilaginous cells are able to synthesize the proteins with a mechanism which is probably similar to that of other cells. Of the different proteins considerable attention is paid to the collagen because of its function.

Concerning the biosynthesis of collagen, a controversial question is whether hydroxylation of proline takes place before proline is incorporated into peptidic linkage or after a microsomal RNA-bound peptide of considerable size has been formed (Gross 1964).

According to Manner and Gould (1963) and Jackson et al. (1964), quoted by Delbrück (1964), it seems that proline is hydroxylated when bound to soluble RNA or when it is linked in a peptide bound to soluble RNA. The hydroxylation is strictly oxygen dependent (Prockop et al. 1962, 1963, Flanagan and Nichols 1962, 1964).

Other metabolic processes. Many other metabolic processes are demonstrable in cartilages: Table XX gives a summary of the enzymes which catalyse general metabolic pathways. Unfortunately, we have no time to discuss each one, outlining the influence of hormones and vitamins, among which special mention must be made of Vitamin C. This is involved in biosynthetic processes of connective tissues and was discovered and studied first in this country by Szent-Györgyi (1932).

Bone metabolism. This has been studied both with biochemical and histochemical procedures. Unfortunately, the biochemical methods encounter serious difficulties in assessing the metabolic contribution of the different cell types (osteoblasts, osteocytes and osteoclasts).

It may be supposed that in these cells all the principal metabolic pathways are possible; the differences are actually more quantitative than qualitative.

TABLE XX

Miscellaneous enzymes of preosseous cartilage and bone tissue

Alkaline phosphatase	Robison (1923) Camurati et al. (1955) Bourne (1956)
Alkaline phosphatase (bone)	Vaes (1964)
Acid phosphatase (bone)	Kochakian (1952) Vaes (1964)
Sulphatase (periosteum)	Follis (1951)
Aryl sulphatase (ep. cart.)	Zambotti et al. (1957)
Desaminase	Lutwak-Mann (1940)
Peroxidase	Giunta (1954)
Transketolase (ep. cart.)	Bolognani et al. (1958)
Phosphoamidase (bone)	Shajowicz et al. (1964)
Glutamic oxalacetate transam. (bone)	Tessari (1960)
Glutamic oxalacetate transam. (ep. cart.)	De Bernard et al. (1955)
Alcohol dehydrogenase (bone)	Herman-Erlee (1962)
Glucose-6-P-dehydrogenase (bone)	Hekkelman (1963) Balogh et al. (1961)
Glucose-6-P-dehydrogenase (cart.)	Kuhlman (1960) Balogh et al. (1961)
6-P-gluconic acid dehydrogenase (bone)	Hekkelman (1963)
β-galactosidase (bone)	Vaes (1964)
β-glucuronidase (cart.)	Lorenzi (1952)
β-glucuronidase (bone)	Vaes (1964) Cabrini (1961)
Hexosamine synthetase (cart.)	Castellani et al. (1956)
Hyaluronidase (bone)	Vaes (1964)
UPDG dehydrogenase (cart.)	Zambotti et al. (1957)
UPDG glycogen transpherase (cart.)	Tettamanti et al. (1962)
Lipase (cart.)	Barbieri (1958)
Sulphate kinase (cart.)	D'Abramo et al. (1957)
DNAse and RNAse (bone)	Vaes (1964)
Adenosin deaminase (cart.)	Tancredi et al. (1958)
5-nucleotidase (cart.)	Reis (1950)
ATPase (cart.)	Cartier (1952)
AMP and IMPase (cart.)	Tancredi (1958)
Inosine nucleotidase (cart.)	Tancredi (1958)
Catalase (bone)	Vaes (1964)
Cathepsin (bone)	Vaes (1964)
Carbonic anhydrase (ep. cart.)	De Bernard et al. (1955*b*)

Glycolysis. In the bone tissue an active glycolysis takes place as demonstrated by Lasking and Engel (1956) and Borle et al. (1960).

HMP shunt. In the bone tissue the hexosemonophosphate shunt is quite intense. Cohn and Forsher (1962) established this in metaphyseal and epiphyseal bone with differently labelled glucose (^{1}C, ^{6}C and C^{u}). Hekkelman (1963) demonstrated the presence of dehydrogenases which are responsible for starting this cycle (G—6—P dehydrogenase and 6—P—gluconic dehydrogenase).

Citric cycle. In 1952 Dixon and Perkins observed the presence of aconitase and enzyme condensing citrate in bone tissue. Van Reen (1959) confirmed the presence of aconitase and evidenced isocitric dehydrogenase; Hekkelman

(1963) obtained similar results. Outstanding proof supporting the existence of the citric cycle in the bone was furnished by Norman and De Luca (1964) demonstrating the incorporation of labelled acetate in the following organic acids of the bone: citric, alphaketoglutaric, succinic, malic acid.* General details of the enzymes of the citric cycle in the bone are given in Table XVIII.

Protein synthesis and collagen biosynthesis in bone. Obviously the bone tissue cells are able to synthesize proteins and collagen. It seems to be acceptable that the biosynthetic mechanisms involved are substantially the same in this tissue as in other connective tissue sections.

Flanagan and Nichols (1964) studied the incorporation *in vitro* of proline ^{14}C in the metaphysis of 55-day-old rats. Proline is taken up in the cells quickly and is incorporated in the collagen.

Incubating rat metaphyseal fragment with glucose U ^{14}C formed labelled amino acids; among these, there are very highly labelled glutamic acid and alanine. Less labelled amino acids are: aspartic acid, glycine, proline and hydroxyproline. Iodoacetate inhibits collagen synthesis starting from proline ^{14}C.

MINERALIZATION OF OSSIFIABLE CARTILAGE

After the discussion of the chemical composition and the main metabolic processes of cartilage and bone tissue, we must now take into consideration mineralization. Although the data reported give a great deal of information, they are insufficient for explaining the mineralization process satisfactorily.

Once again we encounter the difficulty to understand a life phenomenon on the basis of relative or absolute chemical data of the proteins, fat, sugars, coenzymes, etc. which build up an organism. More important information to explain the mineralization mechanism must be furnished by dynamic biochemistry of the ossifiable cartilage and of bone tissue. Unfortunately, such information is still inadequate, and this justifies the existence of several hypotheses which reflect the efforts made by different authors, e.g. Robison (1923), Glock (1950), Gutman and Gutman (1941), Cartier (1952), Sobel (1955), Glimcher (1960), Fleish and Neuman (1961), Fleish and Bisaz (1963), Urist (1964) and many others, including our school (Zambotti 1957, 1959).

It must be remembered that the main bone-tissue compounds, e.g. Ca^{2+}, PO_4^{3-}, CO_3^{2-}, citrate, collagen, non-collagen proteins, mucoproteins, mucopolysaccharides, etc. are present in many tissues which do not ossify under normal conditions at all. The question is how to explain the fact that under physiological conditions the mineralization takes place only at particular sites. It must be assumed that in the calcifiable matrix a particular

*It should be noted that organic acids are present in the bone in two forms: 1. soluble, metabolically corresponding to that existing in soft tissues, and 2. unsoluble inactive, incorporated in the bone salt. Fumaric and α-ketoglutaric acid are present mainly in the first form (Lees and Kuyper 1957).

condition develops; what is this condition? Does a "local factor" of mineralization exist?

We know many inhibitors of mineralization as pointed out by Sobel and Burger (1954), but probably no single local factor exists which is responsible for the mineralization (Urist 1964).

Phosphate, phosphorylase, citrogenase, coenzymes, phosphoric esters, UTP, ATP, etc. considered individually are indispensable, but are no such factors because they are not specific. The osteogenine of Levander (1938) must not be taken into account.

The most honest answer to the question which are the factors or conditions determining ossification is that we do not know. We only have hypotheses and we will try to summarize them briefly.

First of all, the bone salt is quite complex in composition and perhaps

TABLE XXI

Hypotheses on the composition of mineral phase in bones

Formula	Reference*
Mixture of Ca_3PO_4 $Mg_3(PO_4)_2$, $CaCO_3$ CaF_2, etc.	
$Ca_5H_2(PO_4)_2$	Berzelius (1845)
$Ca[(O,PO_3Ca)_2Ca]_3CO_3$	Hoppe-Seyler (1862) Werner (1906) Gassman (1937)
$Ca(OH)_2[Ca_3(PO_4)_2]_3$ + carbonate and bicarbonate	Klement (1929)
$CaCO_3[Ca_3(PO_4)_2]$ n = 2 or 3	Bogert and Hastings (1931)
$(OH)_2Ca_6 \cdot [(P,C)O_4]_6(CaC)_4$	Grunner et al. (1937)
$Ca(OH)_2 \cdot [Ca_3(PO_4)_2]_3$	Bale (1936) Hodge et al. (1938) Thewlis et al. (1939)
$CaCO_3 \cdot n[Ca_3(PO_4)_2]$ n = 1·86—3·33 m $CaHPO_4 \cdot$ n $[Ca_3(PO_4)_2] \cdot CaCO_3$	Sobel et al. (1945)
$(Ca, Mg, Na)_9 \cdot (PO_4, CO_3)_6 \cdot (H_2O)_2$	Hendricks and Hill (1947)
$Ca_3(PO_4)_2 \cdot H_2(OH)_2$ + $CaCO_3$, $MgCO_3$, etc.	Dallemagne (1947)
$Ca_{10}(PO_4)_6(OH)_2$ + PO_4, CO_3, Mg, Na citrate	Hendricks and Hill (1950)

* See in Armstrong (1950)

not completely clarified; this is also revealed by numerous formulae which have been proposed. The main formulae are summarized in Table XXI.

The first problem we will consider is the solubility product of tricalcium phosphate which is the main component of bone salt. Tricalcium phosphate precipitates only if the solubility product is superated. (The solubility product of $Ca_3(PO_4)_2$ is very small.)

In order to explain how to overcome the solubility product, Robison (1923) drew attention to the alkaline phosphatase. Such an enzyme hydrolyses glucose—6—P to glucose and phosphate, increasing the phosphate ion concentration until it exceeds the solubility product of $Ca_3(PO_4)_2$; this salt would, therefore, precipitate at sites where phosphatase is particularly active. This hypothesis does not explain the inhibiton of calcification *in vitro* by some enzyme poisons, such as monoiodoacetate fluoride, etc. which inhibit calcification even at a concentration far too low to inhibit alkaline phosphatase or to interfere with the precipitation of calcium phosphate.

Glock (1940) suggested that the phosphate ions for the formation of the bone salts are obtained from the phosphate esters formed through glycolysis. Gutman et al. (1942) provided experimental evidence supporting Glock's observations. Gutman (1946) and Gutman and Yu (1950) studying calcification in the presence and absence of fluoride (which inhibits enolase) put forward the hypothesis that the phosphate donor could be the phosphopyruvate. Gutman and Yu (1949) also examined the possibility that ATP might act as phosphate donor, but they did not obtain definite results, probably because the biological material they used, cartilage from rickety rats, was not suited for these researches.

Later researches of Cartier (1950) and Cartier and Picard (1955) on the cartilage of normal animals (ram embryos) indicated that the phosphate donor is probably ATP. Such compounds which are formed through glycolysis, tricarboxylic acid cycle and fatty acid oxidation process are donors of phosphate or pyrophosphate, and according to observations they have been found joined to the protein matrix of the bone. Pyrophosphate in bones was established by Perkins and Walker (1955) and its presence was confirmed by Cartier (1957).

Several groups of organic matrix can bind phosphate or pyrophosphate: polar groups of the polypeptidic chains according to Cartier and Picard (1955) and aminic group according to Solomons and Irving (1956) and Wuthier et al. (1964).

According to Solomons and Irving (1956), the number of free amino groups of human dentin is proportional to the degree of mineralization of the tissue. These observations suggested a working hypothesis that transpyrophosphorylation reaction probably takes place in ossifying cartilage between ATP and free amino groups of collagen according to the scheme:

$$\text{Collagen}-NH_2 + \text{ATP} \dashrightarrow \text{collagen}-NH-PP+AMP$$

Since this reaction is not frequent in biochemistry, it may constitute a "local factor" sufficiently typical of preosseous cartilage, determining perhaps the calcaffine property of preosseous matrix (Cartier and Picard 1955).

Beneath the transpyrophosphorylation process, we admit that transphosphorylation also takes place as follows:

$$\text{Collagen}-NH_2 + ATP \dashrightarrow \text{collagen}-NH-P + ADP$$

In these reactions phosphate or pyrophosphate are admitted to be joined with $-NH_2$ group in order to explain the bound $-NH_2$. However, Krane and Glimcher (1965) found phosphate joined to serine (—OH group). These reactions outline the role of collagen in the mineralization process. In fact, it is generally accepted that the collagen combined with phosphate participates in the nucleation processes, i.e. in the formation of crystal seed which will grow up by epitaxis afterwards. Glimcher et al. (1957) deeply studied the function of collagen in mineralization, which they think may be able to induce nucleation of apatite crystals at the macromolecular level of collagen fibrils. By the juxtaposition of certain reactive groups in the collagen fibrils, highly specific zones are built up that may act as sites for nucleation.

Another factor which, according to Sobel (1955) and Zambotti (1957), has an outstanding function in the mineralization of cartilage is the chondroitin sulphate, free or bound to proteins. The importance of this factor results from different experimental data, i.e. that sulphurylated mucopolysaccharides are present wherever calcification takes place: in the epiphyseal cartilage, in the bone, in the dentin, in the enamel and in the pathological calcification of blood vessels.

Sobel observed a particular behaviour of strontium rickety cartilage towards mineralization agents. This cartilage does not undergo calcification when incubated in a mineralizing solution, unless previously treated with an excess of calcium chloride in order to remove chondroitin sulphate-bound strontium. An analogous blocking of calcification could be obtained by beryllium and other ions, and some organic substances (protamine, toluidine blue, etc.).

Caglioti et al. (1955) and Zambotti (1957) pointed out some years ago that a biochemical basis of nucleation could be formed by a complex between collagen-pyrophosphate-calcium, chondroitinsulphate (Fig. 2).

Recent observations are reported, according to which the MPS hinder mineralization; chondroitin sulphate shields the reactive sites of collagen fibrils which cannot react with pyrophosphate given by ATP.

In vitro researches of Neuman (1960) suggest that collagen is an excellent inducer of nucleation; this property is much more evident if purified collagen is employed. If the collagen should demonstrate similar properties *in vivo*, too, the biochemical study of mineralization should be directed towards the research of factors which control the nucleation in ossifiable cartilage or inhibit them in tissue and organs where mineralization never takes place under physiological conditions. Among these factors which might control and/or hinder nucleation, at present mucopolysaccharides and pyrophosphate are considered (Fleish and Neuman, 1961 Fleish and Bisaz 1963).

The problem of interaction between mucopolysaccharides and collagen must be studied again keeping in mind not only the concentration of the

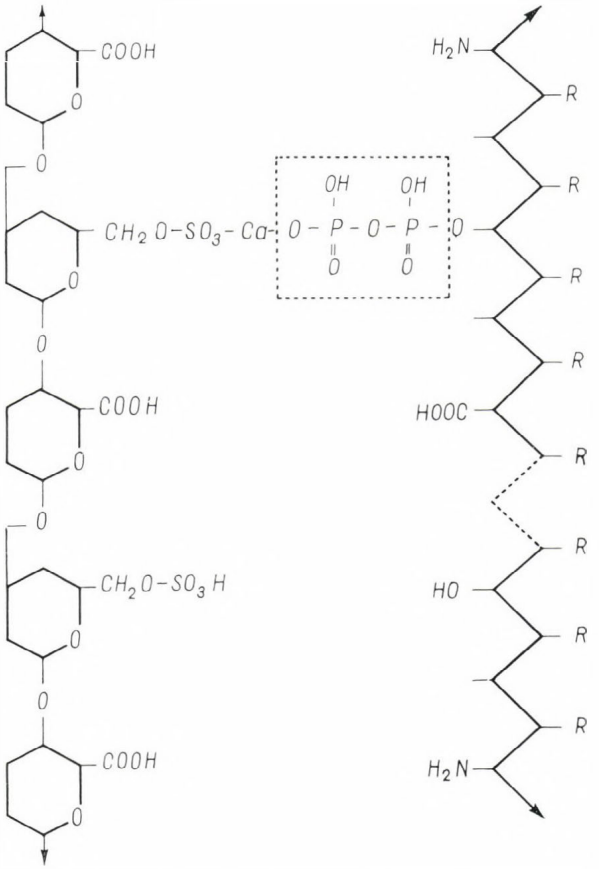


FIG. 2. Possible interactions between CS-capyrophosphate-collagen

mucopolysaccharides, but also their polymerization degree and the different combinations between polysaccharide and proteins. Moreover, it must be borne in mind that mineralization takes place under aerobic conditions which are determined by blood vessel penetration in the mineralization zone with consequent oxygen supply. Aerobic conditions promote the decrease of mucopolysaccharides as demonstrated by Krompecher (1964). This observation is in agreement with the research of Sledge and Dingle (1965) who observed that mucopolysaccharide decreases in the matrix of the limb of chicken embryos cultivated *in vitro* under aerobic conditions.

In order to give an interpretation of the informations already reported and their correlation,we put forward the following scheme of the mineralization process.

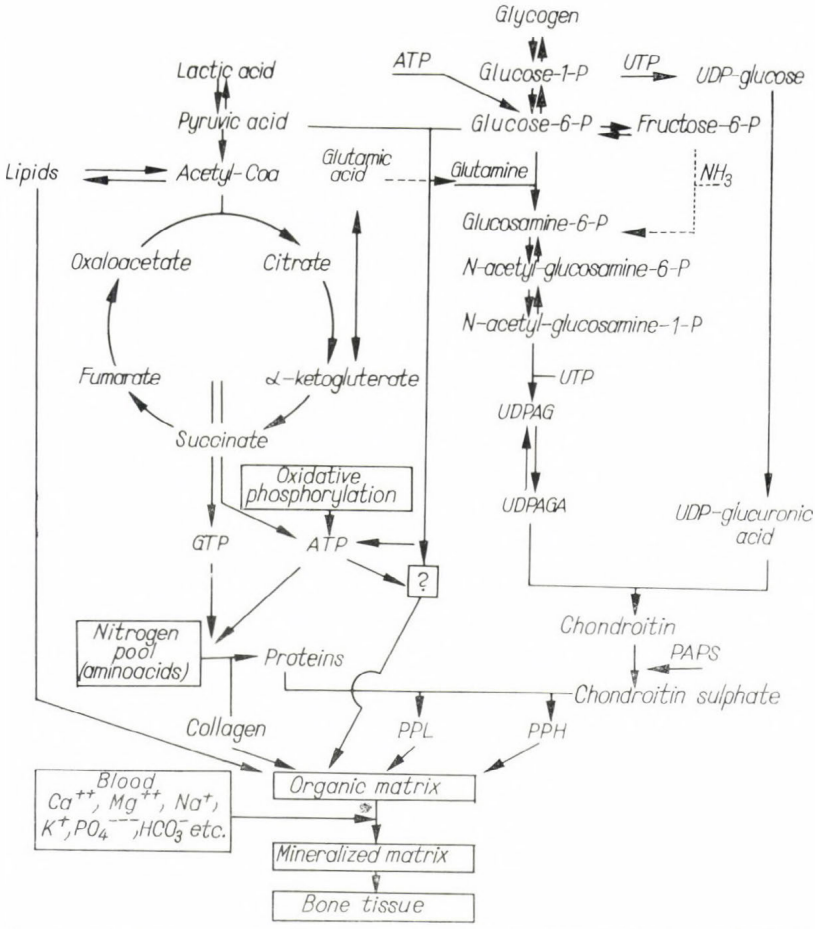


FIG. 3. Attempt to co-ordinate metabolic processes which occur during ossification

1. At the beginning the organic matrix (collagen, MPS, mucoprotein) and high energy phosphorylated compounds, particularly ATP, are synthesized. In this phase we have no nucleation only ion enrichment (Ca^{++} and PO_4^{---} especially) picked up and stored by MPS which showing so many polar groups must be considered an ion exchange resin.

2. Shortly before the deposition of bone salt, the zone where mineralization will take place, become vascularized, i.e. strongly aerobic: oxygen encourages oxidative processes and the production of increased amount of ATP. At the same time oxygen acts upon cartilaginous cells, changing the permeability of the membranes and promoting the outflow of acidic hydrolase (proteases, β-glucuronidases, phosphates, etc; Vaes 1965, Weissmann and Thomas 1963, Woessner 1965). Such enzymes deeply modify the ground substances, probably affecting the protein-polysaccharide

complex. The reactive groups of collagen ($-NH_2$, $-OH$, etc.) are no longer masked and become available to be phosphorylated or pyrophosphorylated by ATP, forming, nucleation centres as already mentioned. At the same time, Ca^{++}, PO_4^{---} and other ions previously stored by polysaccharides become free, and may be arranged around the nucleation centre forming the first microcrystals of bone salt. Such microcrystals grow up by epitaxis.

All these processes are summarized and correlated in Fig. 3.

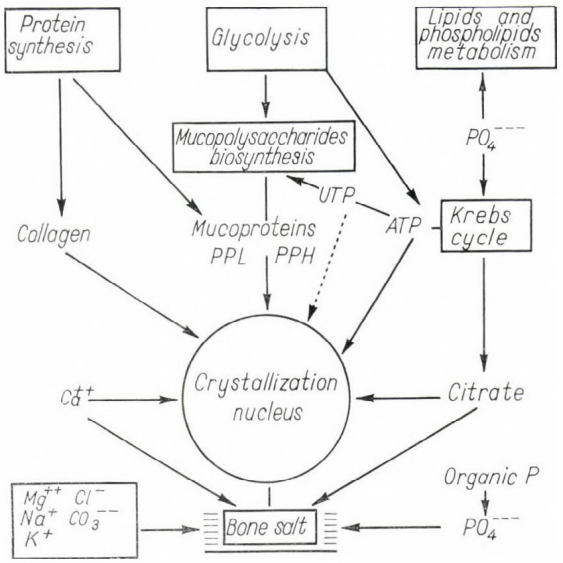


FIG. 4. Attempt to correlate general metabolism of ossifiable cartilage and mineralization process

The interaction between the metabolic processes as illustrated builds up the conditions needed for bone salt formation which are summarized by the hypothesis of the formation of nucleation centre. This centre conditions the formation of the bone salt microcrystal which, thereafter, will grow up by epitaxis at the expense of cations and anions supplied from the blood or produced locally (Fig. 4).

The newly formed bone salt changes under the influence of the ion exchange with surrounding fluids, favoured by the large surface until it reaches the final typical composition. It must be added that the bone tissue is in a dynamic condition, so that it is constantly renewed, although with variable speed depending on the type of the bone, age and other factors.

Obviously, a normal ossification supposes that an equilibrium between different ions and between different metabolites will be reached, and this is strictly related to a balanced course of the general metabolic process and particularly of the mineralizing tissue. The causes capable of modifying such equilibria and interfering with normal metabolic sequences also modify the mineralization process.

We believe that this hypothesis will last little longer than this Symposium, but we shall be pleased if this is so, as it will indicate that we are all making progress.

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Symp. Biol. Hung., 7, pp. 35—56 (1967)

ON THE TEMPORAL COURSE OF WOUND HEALING WITH SPECIAL REFERENCE TO THE SYNTHESIS OF GROUND SUBSTANCE AND FIBRILS

(MORPHOLOGICAL, BIOCHEMICAL AND RADIOCHEMICAL EXAMINATIONS

by

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Wound healing and inflammation have always been in the centre of medical attention and medical investigation. In these processes of the connective tissue, all three components of the connective tissue participate: the cells, the fibrils and the ground substance, forming functional and morphological units with the terminal net of capillaries and nerves. This has been known for a long time for inflammation and since the classic description of Marchand (1924), also for wound healing.

Catabolic and anabolic processes can be distinguished in the course of wound healing. A strict temporal separation is, however, not possible since both of these processes (according to recent findings the anabolic processes as well) set in as early as within the first hour from the onset of wound healing and inflammation, and are carried on simultaneously with varying intensity (depending on the nature and strength of the inflammatory stimulus as well as on local factors of the connective tissue concerned).

In this report we wish to describe the results obtained by our working team, with special reference to our newest findings, and some data of the rather extensive literature on the subject.

By these results given without extensive methodical details, we attempted to elucidate the most important stages in the temporal course of inflammation and wound healing.

Immediately after the onset of inflammation, before any noteworthy morphological changes are recognizable, a primary acidosis that may reach pH values of about 6·0 is found in the irritated or traumatized connective tissue. Such an acidosis was elicited by a skin lesion, the size of a point (insertion of an electrode) in the rat skin (Fig. 1). Normalization occurred within 30 minutes. If the inflammation is caused by other means, e.g. by injection of 0·5 ml of N/10 sodium hydroxide or N/10 hydrochloric acid (Fig. 1), a chemical lesion with a prolonged tissue acidosis is produced. This essential primary response does not depend on the nature of the cause of lesion or inflammation as it can be elicited by physical stimuli or by phlogistics. This response is brought about not only by accumulation of

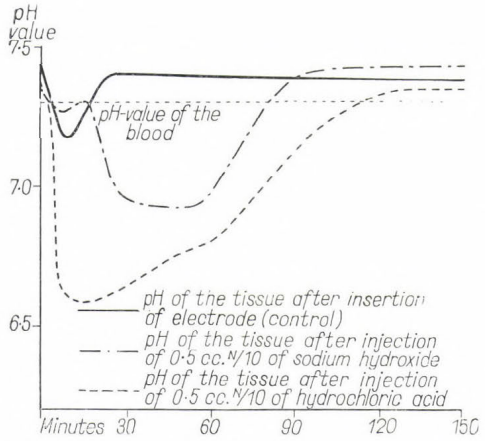


FIG. 1. Primary acidosis in traumatized connective tissue and normalization within 30 minutes compared with stronger reactions

acid metabolites of the connective tissue concerned (e.g. lactate as a result of increased anaerobic glycolysis) and other disturbances of the normal regulation of pH values in the connective tissue by buffer substances occurring here in large amounts (alkaline reserve: bicarbonate phosphate, protein). Schade (1923, 1926) pointed out that the intensity and duration of tissue acidosis (primary acidosis as well as subsequently occurring secondary acidosis) is not conditioned by primary glycogenolysis and secondary increased glycolysis, as was supposed by Frunder (1952, 1953), but by changes of the normal eucolloidal state of the connective tissue. This is the disintegration (*Entmischung*) of the ground substance (Schallock and Schmidt-Matthiesen 1955, Schallock and Lindner 1957). By systematic histochemical, electrochemical and colorimetric examinations (Schweinitz et al 1960, Eckstein et al. 1960) we have

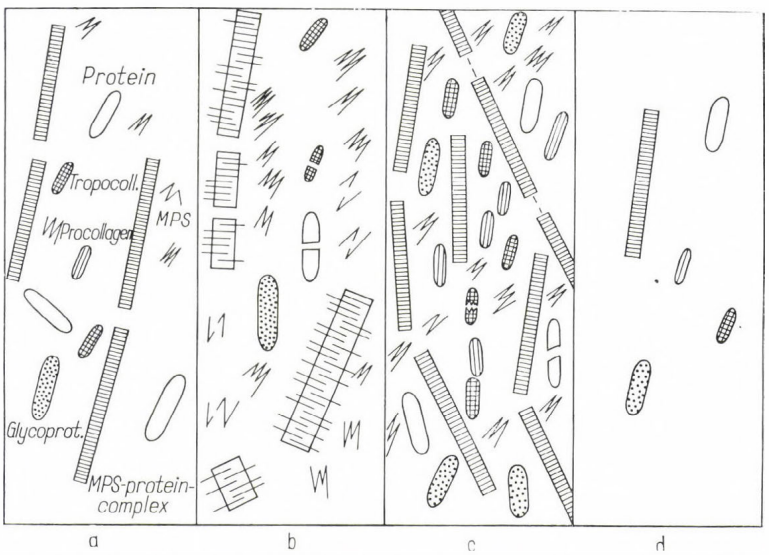


FIG. 2. Normal equilibrium of the components of ground substance, particularly of the mucopolysaccharide-protein complexes (a and b); diagrammatical demonstration of the eucolloidal state. Disintegration of connective tissue *(Entmischung)* (c and d)

established that the changes described in the acid-base equilibrium of the primary response of inflammation and wound healing is due to an increased release of acid mucopolysaccharides of the ground substance.

The course of the process is shown diagrammatically in Fig. 2. In Fig. 2a the normal combination of acid mucopolysaccharides and their protein part is presented. In native form the acid mucopolysaccharides of the connective tissue do not occur in free form but bound to proteins. For the acid mucopolysaccharide chondroitin sulphate it has been proved that about 60 to 62 (even 65) molecules of chondroitin sulphate with an average molecular weight of 50,000 are bound as a salt to a rod-shaped protein nucleus, the share of which is about 20 to 25% of the whole molecule. These mucopolysaccharide-protein complexes are split in parts with every change of the state of the connective tissue, particularly at the onset of inflammation and wound healing. During the disintegration of the connective tissue the mucopolysaccharide-protein complex series and chains of different length fall to pieces followed by the so-called disaggregations and depolymerizations, denaturations, separation of esters, as well as mucolyses and proteolyses accompanied by a general increase and uptake of water. These processes are demonstrated diagrammatically in Fig. 2b, and the picture of the stages of granulation (Fig. 2c) and scarring (Fig. 2d) are presented.

These processes are fairly demonstrable because the acid sugars, as a result of the release of mucopolysaccharides from their protein binding, become available in higher amounts for the demonstration with the corresponding cationic stains. This holds also for the findings reported by other authors (Campani and Reggianini 1950, Ehrich 1956, etc). A genuine increase of the amount of mucopolysaccharides and ground substance is demonstrable histochemically, but in later stages of inflammation and wound healing a precise quantitative assay has been possible with biochemical and radiochemical methods only (Lindner 1957, 1959a, b, c, 1960, 1961/1962, 1962a, b, 1963, 1964a, b, c, 1965a, b, Lindner and Gries 1961, Linder and Schmidt 1964, etc.). The newest findings of our working team indicated, however, that in the first 30 minutes from the beginning of inflammation an increased incorporation of ^{35}S-sulphate may occur which at first probably does not represent a genuine increase of the amount of mucopolysaccharides, but merely an increase of sulphation of mucopolysaccharides in the course of the primary "disintegration process" mentioned above.

In these primary processes of the ground substance, the cement substance between the sub-units of the connective tissue fibrils is likewise involved. As it is known, the collagen fibrils show an hierarchic structural arrangement, beginning with the helical torsion of three polypeptide chains in the protofibril up to the corresponding interlacing of fibrils and fibre bundles. These fibrils and sub-units of fibrils are surrounded by cement substance. In polarized light (Lindner 1959a, b) pools of cement substance, arranged in forms of pods, were seen between the single turns. During the primary processes of distintegration, i.e. due to changes of the surrounding medium, these interfibrillar and perifibrillar pods of cement substance become swollen, their physicochemical relations to the fibrils and fibril-units are

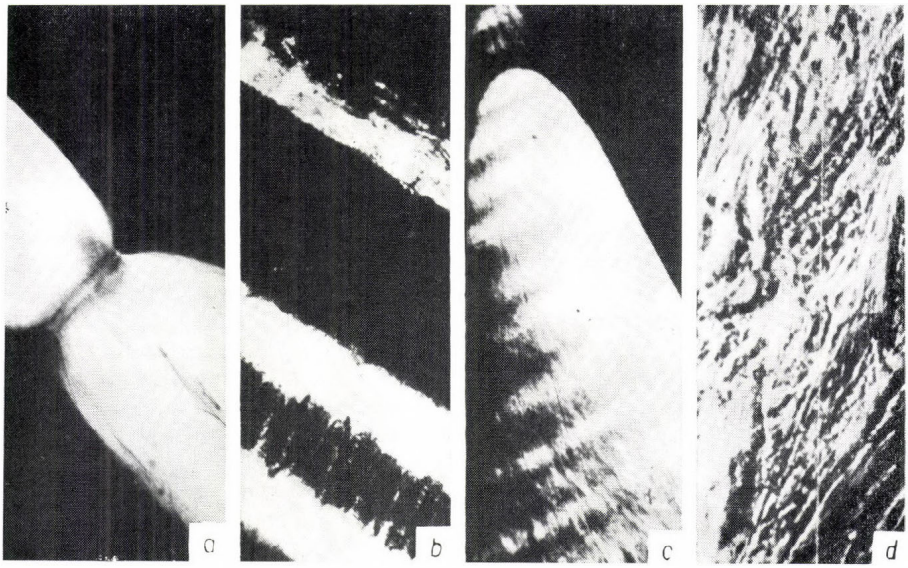


FIG. 3. Swelling of native filaments of the rat tail tendon fibres after incubation with a solution of chondroitin sulphate (a, b c and d)

disturbed and, consequently, these fibril structures become uncoiled. Our detailed examinations on this subject were carried out in the usual way by employing native tail tendons of rats.

Figure 3 illustrates the most important processes occurring in the first phase of inflammation and wound healing. Figure 3a shows that even a few minutes after incubation *in vitro* of native fibrils of rat tail tendon with a solution of chondroitin sulphate, an enormous swelling of the fibrils occurs, chiefly owing to water uptake, accompanied by the known lacing by circular fibrils. Next the ensuing contraction of the fibrils is seen (bottom of Fig. 3b), and a corresponding fibril before contraction, at the beginning of swelling (top of Fig. 3b). We find the periodicity of the swollen fibril, dependent on the known structure, as visible under the polarizing microscope (Fig. 3c), and Fig. 3d shows the typical metachromatic reaction of the interfibrillar cement substance of the disintegrating fibril, stained with toluidine blue (dark spots on the picture) as seen by phase-contrast microscope. It has been shown by us and other authors that the processes *in vivo* occurring during primary disintegration of the ground substance at the beginning of inflammation, correspond to these processes observed in model experiments *in vitro*.

In inflammation and wound healing these processes lead to swelling, instabilizing and unmasking of all the collagen fibrils visible under the light microscope, and in the further course they bring about degeneration products and, thus, denaturation, since native collagen fibrils are extremely susceptible to physical and chemical changes of the surrounding medium.

The processes described are the result of such changes and lead directly to the formation of denaturation products of collagen, by which the way to the degradation of collagen fibrils is prepared. As it has been shown by systematic examinations (Gries and Lindner 1960, 1961, 1963a, Gries et al. 1962, Gries 1965), the degradation of collagen in man and other mammals is essentially a two-step mechanism, since there is no specific collagenase:

1. denaturation of collagen which, because of its structure, is particularly susceptible to mechanical, chemical and thermal influences active in inflammation and wound healing;
2. degradation of denaturated collagen by ubiquitous proteases, such as trypsin, chymotrypsin, fibrinolysin, cathepsin and others.

In this manner the catabolic processes of inflammation and wound healing (regarding degeneration processes see Doerr 1957) approach their climax: degradation of ground substance and fibres, increased by exudation, release of fibrin, leucocytosis, etc. intensified by serum enzymes and by the simultaneous activation and neoformation of enzymes in the emigrated leucocytes and in the resident activated and proliferating connective tissue cells, by intracellular and extracellular digestion, etc. These well-known details remarkably complemented by recent investigation will not be described here. The same holds as regards the extensive complex of cellular modulation, proliferation and differentiation, the processes of terminal circulation, the involvement of terminal reticulum etc. and the effect on inflammation of substances released during the catabolic processes mentioned (histamin, H-substances, etc.). These questions have been examined separately in recent morphological and biochemical investigations on inflammation (Maximow 1902, Marchand 1924, Ehrich 1956, Meier 1959, Lindner 1957, 1959a, b, c, 1960, 1961/1962, 1962a, b, 1963, 1964a, b, c, 1965a, b, Lindner and Gries 1961, Lindner and Schmidt 1964 and others).

The purpose of our investigations was to point out how soon after the onset of inflammation and wound healing anabolic and catabolic processes set in, combine and become demonstrable by recent comparative morphological, biochemical and radiochemical methods. In the temporal course of wound healing and inflammation, the anabolic processes were found to overlap the catabolic processes, described above, even in the first hours. The activation and synthesis of enzymes have already been mentioned. They are based on the synthesis of RNA with syntheses of the required matrix ribonucleoproteids setting in within the first hours after the onset of wound healing and inflammation.

Figures 4 and 5 show this increase of RNA demonstrated by classic staining procedures; in this case using methyl pyronin in activated and proliferating mesenchymal cells during the early stage of inflammation, and in fibroblasts with distinct formation of fibres, during the later stage of inflammation (this demonstration of RNA alone implies an increase of RNA with increased cellular function and not a specificity of certain cellular differentiations, e.g. of plasma cells). Recently, these primary anabolic processes have been localized histotopologically in the involved cells of the granulation tissue, at the beginning of inflammation and have partly

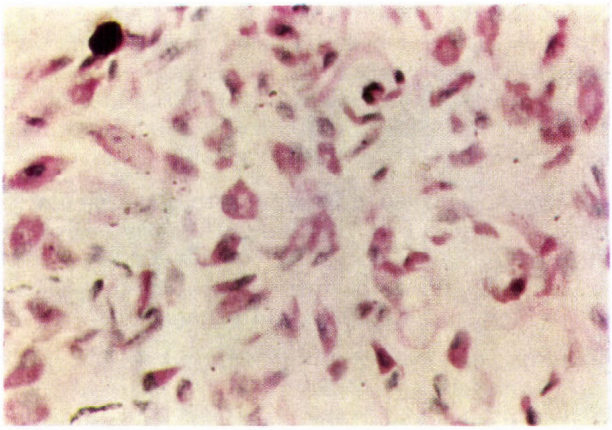


FIG. 4. Increase of RNA in activated and proliferated mesenchymal cells during the early stage of inflammation

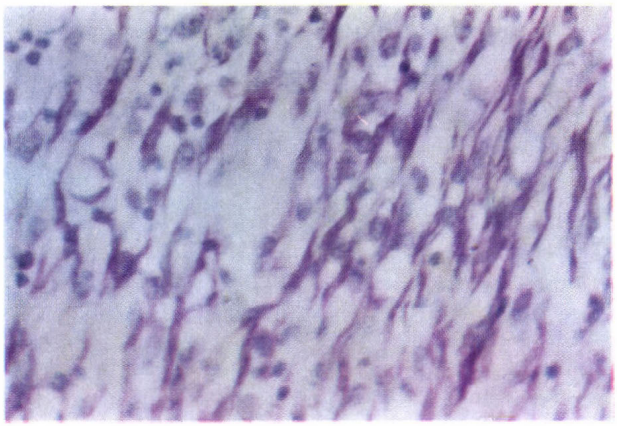


FIG. 5. Increase of RNA in activated and proliferated mesenchymal cells and in fibroblasts during the later stage of inflammation

been demonstrated quantitatively by autoradiography using nuclear components (labelled nucleotides) and by biochemical determinations of the RNA and DNA content.

Thus, the increase of RNA is the preliminary condition for enzyme synthesis, as well as for the formation of specific products (in this case that of components of ground substance and fibres in fibroblasts). The enzyme synthesis following the increase of RNA is now demonstrable with recent enzyme histochemical methods even within the first 2 to 3 hours after the onset of wound healing and inflammation.

Figure 6 shows the highly interesting increase of β-glucuronidase activity, usually demonstrable within two hours after the beginning of inflammation and wound healing, in various proliferating connective-tissue cells of the inflamed area (in this case it refers to rat skin stained by 8-hydroxyquinoline azo-dye coupling method); it may be demonstrated with other procedures, particularly by the method of Fischman and others. This enzyme is of great interest, since it is involved in the degradation of connective-tissue components that were released during primary disintegration of

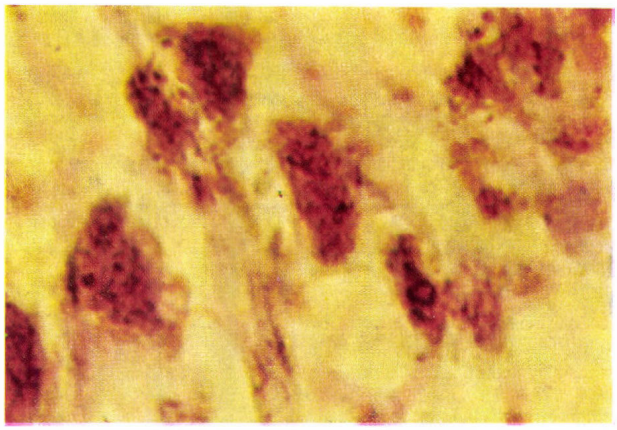


FIG. 6. Increase of β-glucuronidase activity 2 hours after the beginning of inflammation and wound healing in various proliferated connective-tissue cells (stained with 8-hydroxyquinoline azo-dye coupling method)

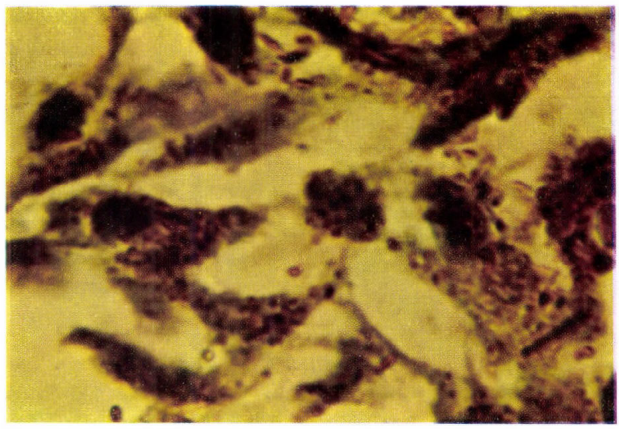


FIG. 7. Increase of aminopeptidase activity in fibroblasts of granulation tissue (stained with azo-dye coupling method)

the ground substance. The same holds for the enzyme aminopeptidase demonstrated in mesenchymal cells, particularly in fibroblasts of granulation tissue, and likewise stained with the azo-dye coupling method (Fig. 7).

The findings obtained in granulation tissue by these newer enzyme-histochemical procedures are summarized in Fig. 8. From this figure it appears that under similar conditions the demonstrability of enzymes in resting connective-tissue cells is reduced, minimal or nil, while the activated cells contain hydrolytic enzymes in an increasing degree as oxydoreductase, and in the further course, lyases and syntheases, too. From the original colour scale, the intensity of the functional increase of enzyme activity of the connective tissue in inflammation and wound healing can be fairly well recognized. In this respect we wish to point out that the enzyme pattern of macrophages and giant cells are rather similar which is further evidence of the fact that also giant cells possess a remarkable metabolic activity and co-operate in the degradation of dissolved tissue material and of foreign substances involved in inflammation and trauma. Moreover, it is clear that connective-tissue cells possess enzyme patterns comparable to those of

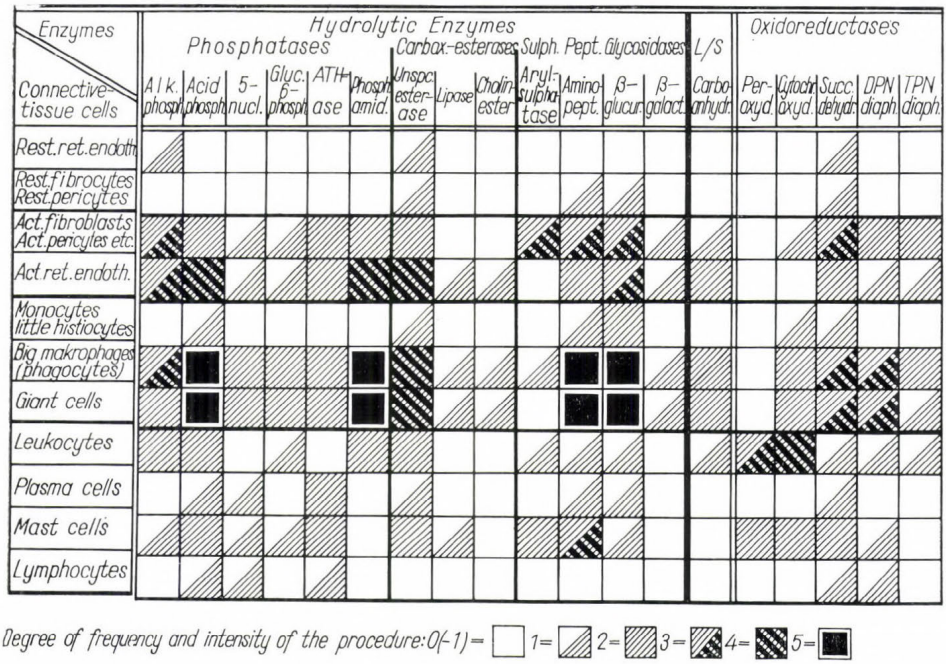


FIG. 8. Comparison of the demonstrability of enzymes in resting connective-tissue cells and in activated cells

epithelial cells, they have a remarkable metabolic activity and output. The prospective potencies of the connective tissue are remarkable. From these examinations it can be concluded that the function and output of the connective-tissue cells are more differentiated than their shape. The summarized enzyme-histochemical results show that qualitative and quantitative differences in the enzyme patterns of certain connective tissue cells can be differentiated. According to these results, the existence of an inductive or adaptative enzyme synthesis in the connective tissue is highly probable. This synthesis takes place in the course of intracellular processes of catabolism, transformation and anabolism with mesenchymal phagocytosis, during inflammatory or post-traumatic tissue cleaning.

Numerous authors reported data on this subject, e.g. French and Benditt (1954), Gedigk and Bontke (1956, 1957), Raekallio (1960), Cabrini (1961), Lindner (1957, 1959a, b, c, 1960, 1961/1962, 1962a, b, 1963, 1964a, b, c, 1965a, b), Lindner and Gries (1961), Lindner and Schmidt (1964). The azo-dye coupling procedures, preferred by us for the demonstration of hydrolytic enzymes, are better for electron-microscopical intracellular localization of the enzymes (for electron-microscopic enzyme histochemistry see Gusek and Lindner 1959).

As has been mentioned before, these enzyme syntheses as well as the neoformation of specific fibroblast products, i.e. ground substance and

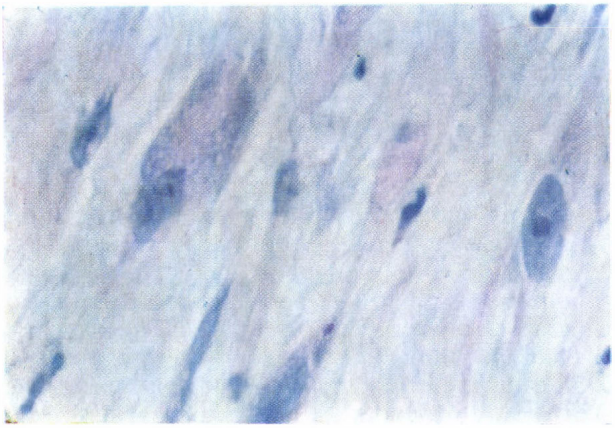


FIG. 9. Demonstration of polysaccharide-protein complexes in fibroblasts of granulation tissue (stained with Astra blue —PAS combination)

fibres, are induced by syntheses of matrix RNA.Thus, we approach—without reporting further details on primary and secondary alterations initiating inflammation—the process termed as third phase of inflammatory proliferation. This we describe in detail since important new results have been obtained recently concerning the early beginning of these synthetic processes and their temporal and causal interdependence. These decisive synthetic and anabolic processes in inflammation and wound healing are not sufficiently demonstrable with component-histochemical procedures. This is evidenced by Fig. 9 showing fibroblasts of granulation tissue stained by the combination of Astra blue and PAS. Polysaccharide-protein complexes demonstrable with various staining procedures (1962b) may be newly formed in diffuse or granular form in various connective-tissue cells, but also may be phagocytized material which cannot be differentiated with these histochemical procedures. According to our detailed examinations, this holds for various connective-tissue cells and not only when anabolic processes predominate (both *in vivo* and *in vitro).* It has even been found that particularly clear demonstrations of polysaccharide-protein complexes obtained with specific histochemical procedures in connective-tissue cells more frequently indicate catabolic processes in phagocytized material than processes of neoformation. This is a definite restriction of the findings obtained first by Gersh and Catchpole (1949). By electron-microscopical examinations it was demonstrated that catabolic and anabolic processes, e.g. phagocytosis and fibre formation can occur simultaneously in fibroblasts (Ross and Benditt 1962). For various reasons, the same certainly refers to the synthesis of the ground substance, although this could not yet be proved unequivocally by electron microscopy (Gries and Lindner 1963b). For the decisive questions of the neoformation of connective tissue, especially the elucidation of temporal and causal interrelationships of ground substance and fibre formation, first of all quantitative results are required. Therefore, parallel to our morphological examinations, we carried out (Gries et al. 1962, Gries and Lindner 1963a, b, c, d, e, Lindner and Gries 1961, Lindner 1961/1962, 1962a, b, 1963, 1964a, b, c, 1965a, b) biochemical and radiochemical analyses of granulation tissue (as neoformation of connective tissue).

Biochemical results. Figure 10 shows the normal temporal course of ground substance and fibre formation obtained on the model of inner wound healing, i.e. the cotton-pellet test. The upper curve represents the result of hydroxyproline assay according to Stegemann, i.e. the assay of amino-acid specific of collagen which is used for the quantitative assay of collagen synthesis. Below it the results of component analyses of ground substance, obtained during its synthesis, are indicated, namely uronic acids (according to Dische), amino sugars (according to Boas), organic sulphate (according to Dodgson and Spencer), supplemented by determinations of chondroitin sulphate (according to Dittman and Cremer) and of methylpentoses (according to Dische and Shettles) not indicated in this figure.

The most important result of these examinations is that, under normal conditions, the first step of neoformation in the granulation tissue is the synthesis of ground substance which is followed by the formation of fibres. Such is the temporal relation of these syntheses. Their causal relation is, however, much more complicated.

For the further elucidation of the temporal and causal interdependence of the synthetic steps in the granulation tissue, comparative examinations were required. This is the theoretical reason for examining the influence of various substances on wound healing in the compass of basic research. The results are of essential importance in clinical and practical medical work, and concerning the effect of drugs used before, during, or after surgical interventions on wound healing.

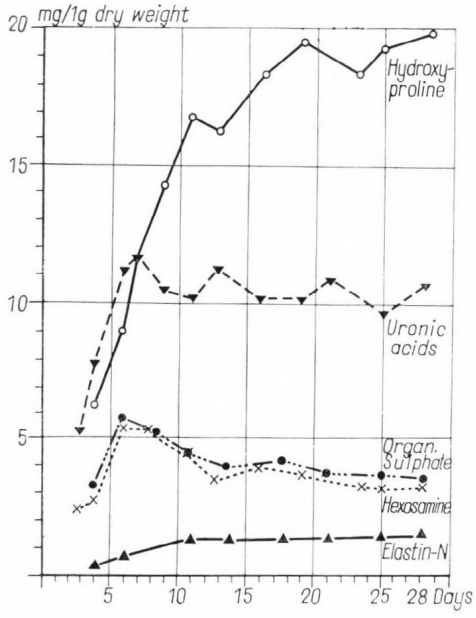
Figure 11, compared with Fig. 10, shows the the effect of cortisol and oxyphenbutazone (Tanderil) on this experimental model of inflammation and wound healing. It is apparent that the formation of collagen is significantly inhibited by the selected concentration of Cortisol (200 mg/kg body weight of the rat), while the degree of inhibition effected by Tanderil, the first metabolite of phenylbutazone (Butazolidine), is smaller. Compared with the control the formation of ground substance shows a slight increase under these experimental conditions. The radiochemical examinations confirmed these results.

Figure 12 presents a comparison of the curves, described above, i.e. the biochemical analysis of fibre formation with corresponding morphological results of normal and influenced fibre formation. There is a clear coincidence in the curves as regards the morphological and biochemical methods.

Figure 13 is a further example of these series of examinations on the effect of some anabolic substances and diuretics on wound healing and inflammation. These substances appear to have an increasing effect not only on general protein metabolism, as it is generally known, but also on collagen formation. Moreover, the diuretics appear to have a clear effect on inflammation and wound healing. This finding suggests that also other substances may be effective in the treatment of inflammation and wound healing by influencing the metabolism of water.

From the facts reported hitherto, it is clear that in normal and influenced wound healing and inflammation, the formation of ground substance is a primary process and that of fibres is a secondary one.

The recognition of the primary importance of the polysaccharides of the



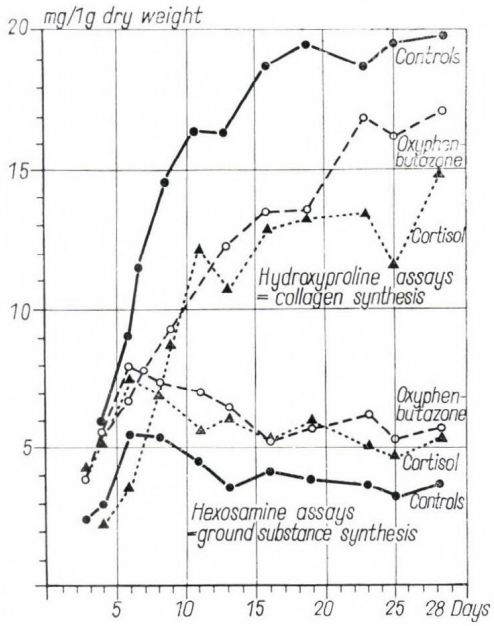


FIG. 10. Normal temporal course of ground substance and fibre formation (biochemical assays)

FIG. 11. Influence of Cortisol and oxyphenbutazone on the temporal course of ground substance and fibre formation (biochemical assays) on the model of inner wound healing

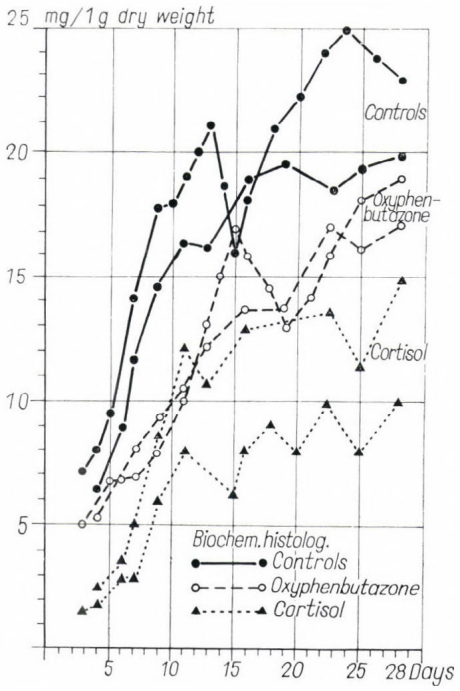


FIG. 12. Influence of Cortisol and oxyphenbutazone on the temporal course of fibre synthesis (results of biochemical and morphological researches)

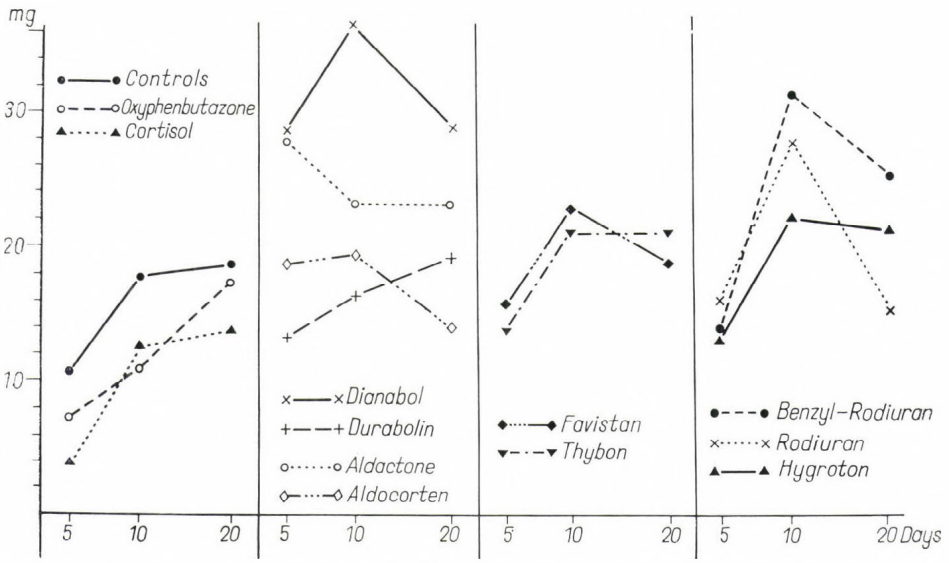


FIG. 13. Influence of some anabolic substances and diuretics on the temporal course of collagen formation (hydroxyproline assay according to Stegemann)

ground substance in the neoformation of connective tissue required further detailed investigation of this primary process with a method that allows the histotopological localization of the details of the ground substance synthesis in histological sections, and performs simultaneous precise quantitative determinations of the same material. These requirements are met by using ^{35}S-sulphate for autoradiography and by parallel quantitative radiochemical determinations of the incorporation rates of ^{35}S.

Autoradiographical and radiochemical results. In the autoradiographical and radiochemical examinations the incorporation rate of ^{35}S is used as a measure of the neoformation of acid mucopolysaccharides and thus, that of ground substance, while the incorporation rate of ^{3}H-proline serves as a measure of collagen synthesis. According to the findings of Dziewiatkowski (1951, 1952, 1962), Boström (1954), Boström et al. (1953), Adams (1959, 1960) Campani and Cortinovis (1960), Curran and Kennedy (1955), Hershberger et al. (1959), Layton (1950), Roseman (1959), Sachs et al. (1960), Salmon (1960) and our working team, the incorporation rate of ^{35}S can be used as a direct quantitative measure of mucopolysaccharide synthesis and so that of the neoformation of connective tissue. As regards the preliminary conditions of this incorporation (elimination of a possible exchange of sulphate groups; [Adams 1959, 1960, Boström et al. 1953, Boström 1954, etc.] and simultaneous synthesis of the polysaccharide and protein parts of this complex, etc.) we refer to our report on autoradiographical and radiochemical studies on the healing of bone fracture in this volume.

Figure 14 is an autoradiogram (stripping-film technique using Kodak AR 10) of a 3-day-old granulation tissue showing that formation of muco-

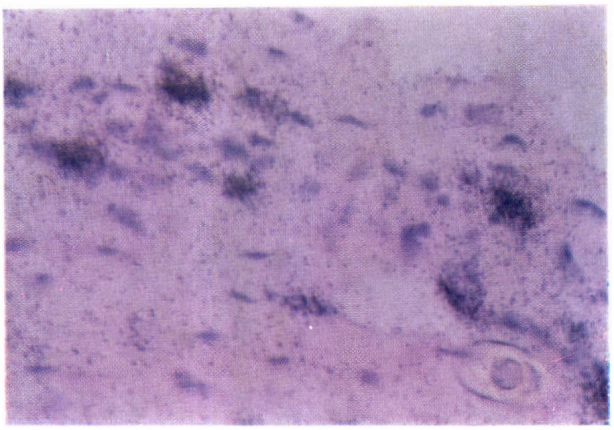


FIG. 14. Autoradiograph of a 3-day-old granulation tissue with demonstration and intracellular localization of the MPS synthesis

polysaccharides by fibroblasts of the granulation tissue does not occur at the same time, but at a given time only part of them are involved in mucopolysaccharide formation and the percentage of the involvement is also variable. This finding is important for various theoretical and practical reasons and will be substantiated by further findings.

First we wish to present the finding obtained with examinations with double labelling (Lindner 1963, 1964b, c, 1965a, b, Lindner and Schmidt 1964) that mucopolysaccharide and collagen syntheses may take place simultaneously in the same connective-tissue cell. In Fig. 15 sections of autoradiograms are shown which were obtained in the cartilaginous tissue

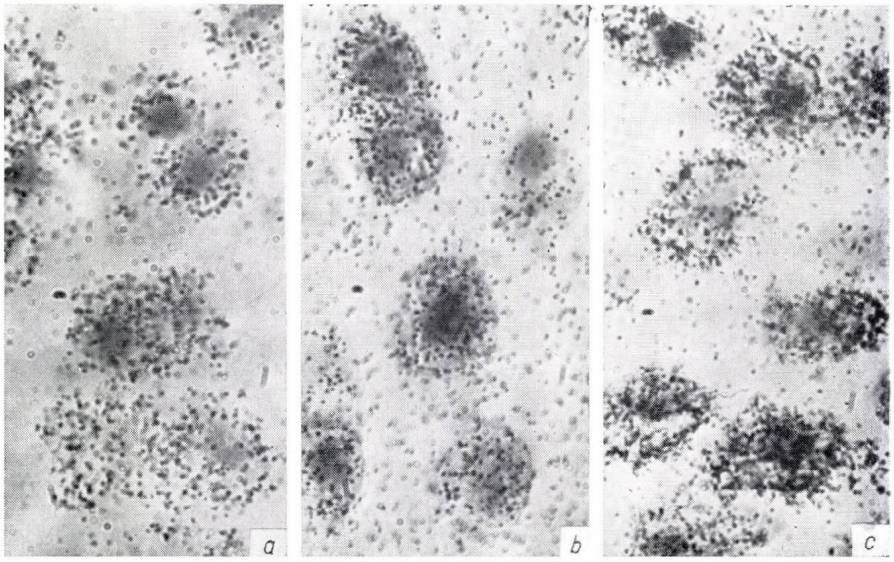


FIG. 15. Autoradiographs of cartilaginous tissue of 8-day-old rats after *in vitro* incorporation of ^{35}S (a), ^{3}H-proline (b), and both of these substances; (c) double-marking technique

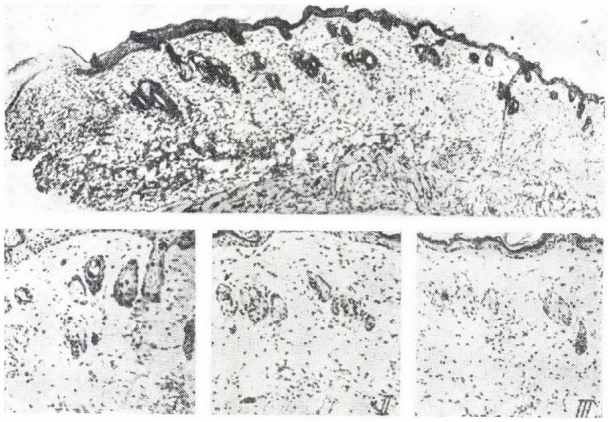


FIG. 16. Experimental wound cut in the back skin of the mouse and 3 skin areas with higher magnification (I, II and III)

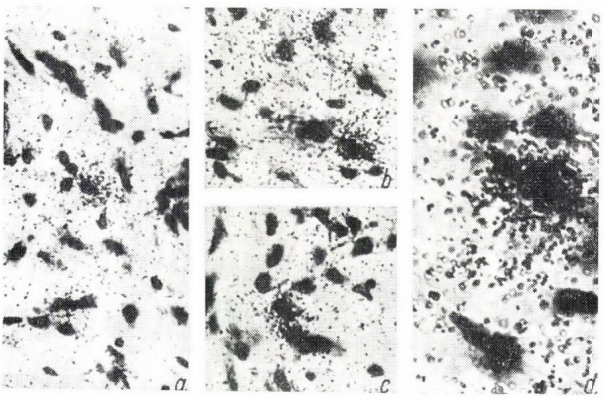


FIG. 17. Fibroblasts of different labelling with various magnifications

of 8-day-old rats after five hours of incubation (with simultaneous determination of oxygen consumption in a Warburg apparatus), after addition of ^{35}S (Fig. 15a), of ^{3}H-proline (Fig. 15b), and after simultaneous addition of both substances (Fig. 15c) demonstrating the simultaneous occurrence of both formation processes in the the same cell.

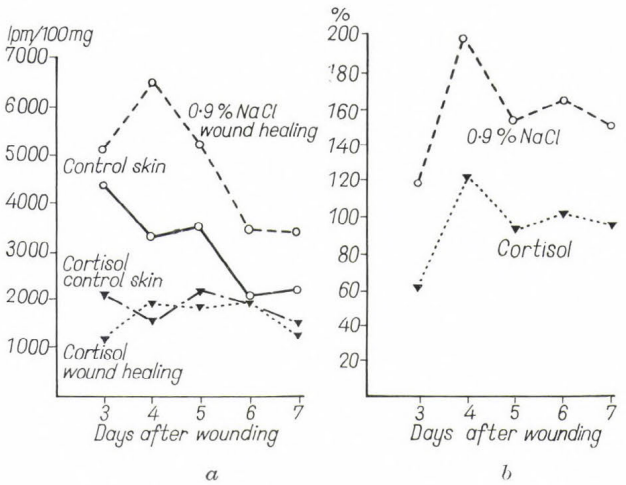
Parallel with the examinations performed on the model of inner wound healing, autoradiographical and radiochemical examinations on experimental open wound healing were carried out as follows: preparation of autoradiograms as reported above, radiochemical examinations of the granulation tissue after 24 hours following a single administration of ^{35}S *in vivo* (intraperitoneal injection), then dialysis of the removed tissue samples, determination of dry weight, wet ashing, precipitation as barium sulphate, counting of impulse rates (minute in methane flow counter, registration in terms of mg dry weight). For further details see our report on callus formation on p. 191.

Figure 16 (upper half) shows the histological pattern of an experimental wound cut in the back skin of the mouse. The cut with the adjacent granu-

lation tissue is at the left margin of the picture. In the lower half of the picture three skin areas are given, in which the number of ^{35}S labelled fibroblasts were counted. Area I is immediately adjacent to the wound, area II starts at a distance of 1 mm from it, and area III is at 2 mm from area I. With this magnification the black grains of the autoradiogram are not sufficiently recognizable but higher magnification shows (in conformity with statements made in connection with Fig. 14) that in uninfluenced wound healing, 2·5 to 3·8% of the fibroblasts are labelled in area I, i.e. in the immediate proximity of the wound, while the corresponding values in area II are 0·8 to 2·3%, and only 0·4 to 1·7 per cent in area III (with 20 or more silver grains per cell in areas II and III, and with 30 to 50 silver grains per cell in area I). These details are illustrated in Fig. 17, where fibroblasts of different labelling are demonstrated with various magnifications. In these examinations only those fibroblasts were assessed which, having 20 or more silver grains per cell, differed significantly from the blank and from the slight labelling of the intercellular substance (as a result of the release of labelled mucopolysaccharides). With medium and higher magnification (Fig. 17a), several labelled fibroblasts appear, while only a few or no silver grains are demonstrable in the rest. In Fig. 17d, single fibroblasts with clear labelling are seen with oil immersion magnification. It should be mentioned that this technique is less suited for photography because, owing to the little focal length, the silver grains in the film are situated above the histological section. From these examinations further evidence was obtained of the fact that only a certain percentage of fibroblasts incorporate ^{35}S-sulphate from mucopolysaccharides at a given time.

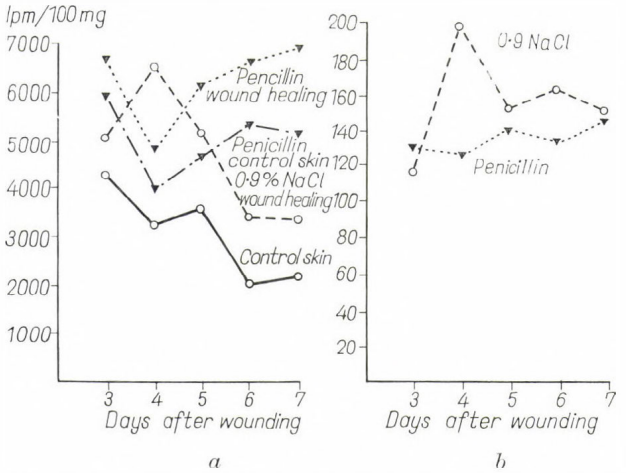
Grasedyck (1965) performed systematical examinations with autoradiographical methods on the influence of various anabolic and catabolic substances on the incorporation rates of fibroblasts. We shall not report the results of his work in detail but give some examples of the influence of treatment on the model of outer wound healing, investigated particularly by Beste (1965) as being of practical importance.

Figure 18 shows the inhibiting effect of *Cortisol* on wound healing. As regards this figure and the subsequent ones, we wish to give the following explanation. Since the substances were given diluted with physiological saline, their effects have been compared with those of physiological saline. The experimental animals were treated daily from the day of wound induction until they were killed; the period of observation reproduced here was seven days. The outer skin wound and the intact control skin of the same animal were examined, so that in each case the ^{35}S-incorporation rate in the granulation tissue and in intact control skin from the third to seventh day after wound induction are given in Fig. 18a for the control (physiological saline) and for the substance applied. On the right side of each diagram we find the relation of the wound to the intact control skin of the same animal. The ^{35}S-incorporation rate of intact control skin on each day after wound induction has been equalled to 100% and then the percentages of the ^{35}S-incorporation rates of the traumatized skin of the same animal have been calculated. As seen in Fig. 18b, far more ^{35}S is



a *b*

FIG. 18. Comparison of the influence of Cortisol and physiological saline on wound healing



a *b*

FIG. 19. Comparison of the influence of penicillin and physiological saline on wound healing

incorporated into the granulation tissue of controls treated with physiological saline than in the intact control skin of the same animal. It is of interest, however, that in animals treated with Cortisol, the incorporation rate of ^{35}S and so the formation of mucopolysaccharides, ground substance and connective tissue in the granulation tissue is only higher on the fourth day than in the intact control skin of the same animal and on the other days it is lower, i.e. more strongly inhibited. From Fig. 18a it is clear that the incorporation rate on ^{35}S, hence the formation of mucopolysaccharides and ground substance in wound granulation tissue and in intact control skin is inhibited by Cortisol.

Figure 19 shows the result of examinations with penicillin demonstrating the influence of antibiotics on wound healing when applied before, during

FIG. 20. Comparison of the influence of Liquemin and physiological saline on wound healing

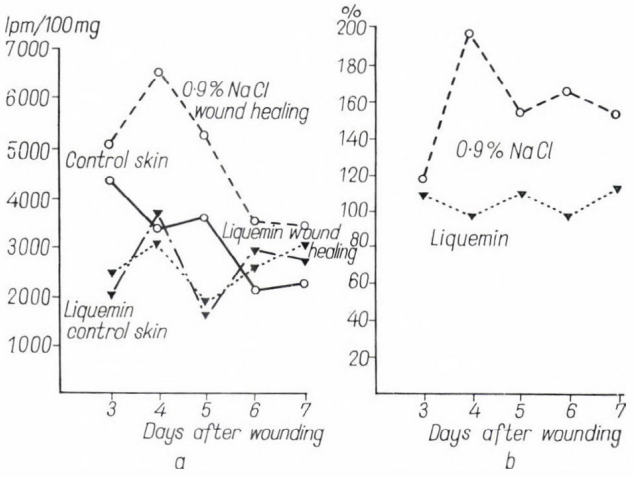
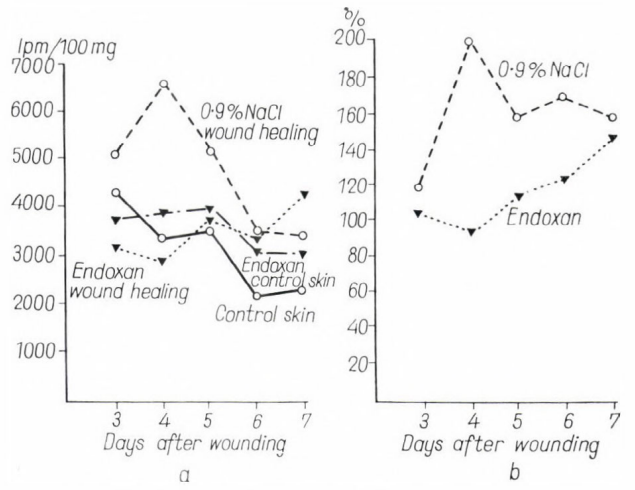


FIG. 21. Comparison of the influence of Endoxan and physiological saline on wound healing



and after, surgical intervention. Penicillin increases the incorporation of ^{35}S in lesioned and intact skin (Fig. 19a) as compared with the controls, whereas the differences of the incorporation rates of traumatized and intact skin are less than in the controls receiving physiological saline (Fig. 19b).

The effect of preoperative and postoperative treatment with anticoagulants is illustrated in Fig. 20 with regard to Liquemin. Liquemin, compared with the controls, leads to an inhibition of the incorporation rate of ^{35}S in traumatized and intact skin (Fig. 20a) and, therefore, the difference of ^{35}S-incorporation rates of traumatized and intact skin after treatment with Liquemin is much smaller than in the controls (Fig. 20b).

Finally, the effect of Endoxan, as an example of postoperative treatment with cytostatics is demonstrated in Fig. 21 with the expected result of inhibition of ^{35}S-incorporation rate, i.e. of the formation of mucopolysaccharides and ground substance in the granulation tissue of wounds, while in the intact skin of Endoxan-treated animals an increase of mucopolysaccharide synthesis is visible, compared with untreated controls.

The last stages of wound healing e. g. contraction (Abercrombie et al. 1954, etc.) are not described here.

For theoretical and practical reasons, described above, numerous authors investigated the effect of various substances on the connective tissue (vitamins, hormones, antiphlogistics, etc.), on the ^{35}S-incorporation rates and on the formation of mucopolysaccharides, ground substance and collagen fibres (Campani und Cortinovis 1960, Agostini et al. 1962, Boström 1954, Curran and Kennedy 1955, Dunphy and Udupa 1955, Hershberger et al. 1959, Layton 1950, 1951, Mancini et al. 1961, Moltke 1957, Pirani et al. 1951, Williamson and Fromm 1954, Williamson und Guschlbauer 1961, Lindner 1957, 1959a, b, c, 1960, 1961/1962, 1962a, b, 1963, 1964a, b, c, 1965a, b, Lindner and Gries 1961, Lindner and Rudas 1961, Lindner and Schmidt 1964, and others). The results vary and are even contrary as shown in Table I.

TABLE I

Influence on ^{35}S-incorporation as measurement of ground-substance synthesis

Hormones		MPS components	
Adrenocort. steroids	−	Glucose	+
Thyroxine	±	Glutamine	+
Insulin	+	Glucosamine	+
Oestrogens	±		
Androgens	+		
STH	+		
Vitamins		**Enzyme toxins**	
		Age	−
		Atherogenic diet	+
		BAPN	±
Vitamin C deficiency	−	X-rays	±
Vitamin A deficiency	+	Antiphlogistics	±

Note: + = increase; − = inhibition.

Table I presents the results obtained by different authors on the increasing or inhibiting effect of certain substances or factors influencing ^{35}S uptake (as a measure of ground substance formation), drawn up by one of our collaborators (Schlieben).

In the examinations carried out by our working team, the same model was used in various dosage series. In addition, examinations were performed

on inner and outer wound healing, on wound healing of cartilage and bone tissue (callus), on normal, influenced and lesioned vascular walls, on other cartilaginous tissues and, mostly, on the development of embryonic connective tissue (Lindner 1959a, b, c, 1960, 1961/1962, 1962a, b, 1963, 1964a, b, c, 1965a, b, Lindner and Gries 1961, Lindner and Schmidt 1964).

The result of these extensive comparative investigations of theoretical and practical importance shows that the effect of substances on the development of connective tissue, particularly on inflammation and wound healing, depends on onset and dosage, that the syntheses of ground substance and fibres can be influenced in various manners and that the temporal and causal interdependences of these steps can be further elucidated by these investigations. Moreover, it shows that the different results found in literature are due to the use of:

1. different models
2. different methods
3. different doses
4. different periods of observation.

Therefore, it is necessary to carry out such extensive comparative examinations as reported here, including the use of temporal series, before any generalization can be made or conclusion can be drawn based on analogy. As

TABLE II

Temporal course of inflammation and wound healing
(onset of the most important stages)

First to fourth hour	Disintegration of ground substance=primary change of physicochemical state with disaggregation, dispersion of colloids, depolymerisation, etc. of mucopolysaccharide-protein complexes, swelling as a result of increased binding of water and other substances with disturbances of isoinia and isotonia: primary acodosis (catabolism)
	Swelling of fibrils (by participation of interfibrillar cement substance in the disintegration of ground substance) (catabolism)
	Unmasking, irritation and swelling of cells with onset of pinocytosis and phagocytosis as well as activation of enzymes of resident connective tissue cells (fibrocytes, histiocytes, cells of the vascular wall, etc.). Synthesis of RNA and adaptive or inductive synthesis of enzymes (catabolism and anabolism)
	Degranulation of mast cells = release of heparin and histamine
	Change of permeability of the capillary wall as a result of participation in disintegration and swelling processes of intercellular substance with influence on capillary function: prestasis, stasis, hypoxia, inflammatory hyperalmia; release of serum (fibrin), erythrocytes, leucocytes, etc. Permeation, exudation, emigration
	Incorporation of ^{35}S (sulphation and synthesis of mucopolysaccharides) (anabolism)

(Table II continued)

Fourth to twelfth hour	Secondary acidosis, continuation of alterations of the ground substance Products of denaturation and degeneration of collagen, destruction by non-specific proteases Increase of intracellular and extracellular catabolism, of glycolysis, proteolysis, lipolysis, etc.
	Incorporation of ^{35}S (sulphation and mucopolysaccharide synthesis) (anabolism)
Twelfth to forty-eigth hour	Increase of initial preponderantly catabolic processes Intensity and duration dependent on site of inflammation as well as on nature, strength and duration of inflammatory stimulus Simultaneous overlapping of catabolic process with primary and secondary anabolic processes: Proliferation of cells (^{3}H-thymidine mitotic rate index), particularly proliferation of fibroblasts, incorporation of ^{35}S and ^{3}H-proline: radiochemical demonstration of ground substance and fibre synthesis
Second to third day	Histochemical and biochemical (uronic acids, hexosamine, mucopolysaccharides) demonstration of ground-substance synthesis
Third to fourth day	Histological and biochemical (hydroxyproline assay) demonstration of fibre synthesis, capillarization
Fourth to sixth day	Shifting of cellular rate with decrease of microphages in favour of maturing fibroblasts; neoformation of mast cells
Sixth to tenth day and later	Decrease of water content, increase of degree of polymerization of ground substance with decrease of histochemical staining properties and increase of physicochemical equilibrium, i.e. of eucolloidal state of ground substance; rate shifting of collagen fractions
	Complete or incomplete restoration of original situation: quantitative and temporal dependence on localization, on nature, degree and exposition of inflammatory stimulus

conclusion and summary of this report, Table II is presented, showing the temporal course of inflammation and wound healing from the onset of the lesion until restoration.

At the same time, Table II summarizes the results of our extensive examinations on inflammation and wound healing and points out the state of our knowledge based on recent comparative morphological, biochemical and radiochemical studies. In this concluding summary the beginning of the catabolic and anabolic processes of inflammation and wound healing are indicated as far as they are demonstrable with recent comparative morphological, biochemical and radiochemical methods. From these the onset of the subsequent stages of wound healing ensuing after wound production or beginning of inflammation, can be recognized, the close sequence of the anabolic and catabolic processes and their close interrelations. Their

temporal and causal combination, their extraordinary quick start and course and the fact that these synthetic processes occur early in each wound healing and inflammation are demonstrated. Moreover, it points out the importance of catabolism and anabolism of the ground substance for the whole further course of wound healing and inflammation. At the same time, it shows in which manner such basic research may serve practical medical work and as to what extent wound healing and inflammation are in the centre of medical research and practice in modern medicine.

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Symp. Biol. Hung., 7, pp. 57—64 (1967)

ON THE BIOGENESIS OF CARTILAGE

by

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The characteristic features of the process of cartilage formation taking place in the adult organism have been studied by authors for a considerable time. Investigations were carried out on an experimental model suitable for such purposes: the regenerating articular surface of the distal part of the femur in the dog. During the investigations, detailed histochemical analyses, determination of carbohydrates, nucleic acid and amino acid metabolism and electron-optical examinations of the differentiating cells have been performed. A total of 800 dogs were used in the experiments.

Semiarthroplasty of the knee-joint, according to Krompecher (1956, 1958a, b), was performed on all the dogs. The operation consisted in opening the knee-joint and removing the cartilaginous articular surface of the distal part of the femur, together with a thin (a few millimetre) layer of the underlying spongious bone. Subsequently, a new articular surface was formed of the spongious bone and the wound was closed.

The granulation tissue developing from the haematoma was found to adhere partly to the bone wound and partly to the articular capsule. As a result of postoperative treatment (moving of the joints from the fifth day on), the originally uniform granulation tissue divides in two: one part adhering to the bone wound and the other to the tissue of the articular capsule. Essentially, our investigations consisted in the examination of these two kinds of granulation tissue, one being termed "granulation tissue adhering to the articular surface" and the other "granulation tissue adhering to the capsule" (Fig. 1). The latter tissue, since no cartilage differentiation occurs in it, served as control. On the other hand, the granulation tissue of the articular surface undergoes a gradual chondrification and regeneration, leading—according to Krompecher's data (1958a, b)—to the development of a new, well functioning and histologically evidenced articular surface in about two years. Our investigations are concerned with the early events of regeneration studied in five stages, selected experimentally. (Hadházy and Oláh 1958):

1. Seven days after operation the bone wound is covered by richly vascularized granulation tissue containing large amounts of fibrin (Fig. 2);
2. Twenty days after operation the granulation tissue is fibrotic, its vascularization is reduced compared with that of the previous stage.

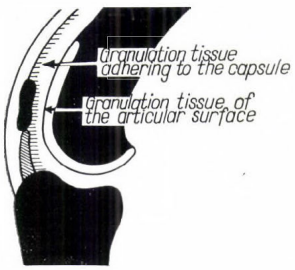

FIG. 1. Schematic representation of knee-joint of dog showing the topographic localization of the granulation tissue originating from a common source. One part of the granulation tissue adheres to the bone wound and later differentiates to cartilage. No cartilage formation can be observed in the granulation tissue adhering to the articular capsule

On the border between the spongious bone and granulation tissue desmal bone building is going on (Fig. 3);

3. Twenty-six days after operation the first cartilage formations appear in the granulation tissue (Fig. 4);
4. Thirty-three days after operation small circumscribed cartilage islets appear in the granulation tissue as if resting on the spongious bone. These islets contain cartilage cells in various stages of differentiation (prechondroblasts, chondroblasts, chondrocytes). Their immediate vicinity is poorly vascularized but in the granulation tissue—at some

FIG. 2. Seven-day-old articular surface. Richly vascularized granulation tissue covered by fibrin on the spongious bone stumps. Haematoxylin-eosin; ×95

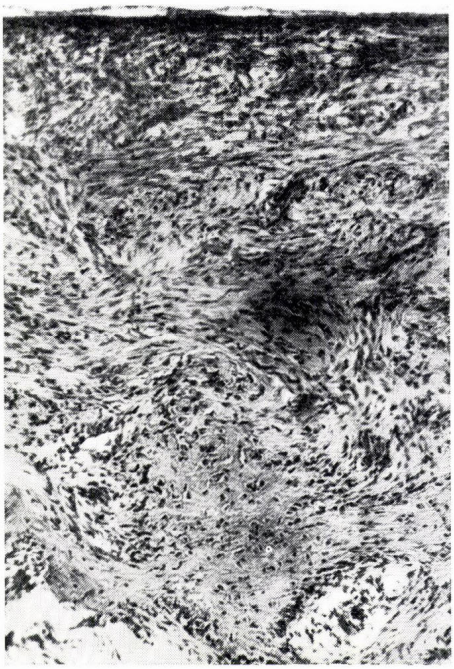

FIG. 3. Twenty-day-old articular surface. Relative decrease in the vascularization of granulation tissue. Desmal bone formation in the vicinity of the spongiosa. Haematoxylin-eosin; ×95

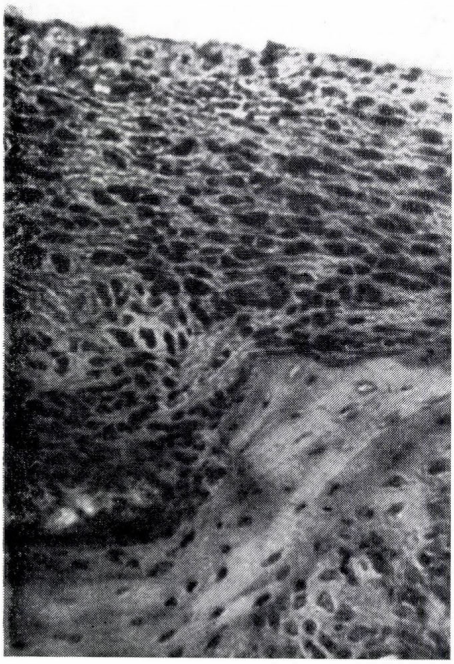

FIG. 4. Twenty-six-day-old articular surface. Incipient cartilage differentiation. Haematoxylin-eosin; ×370

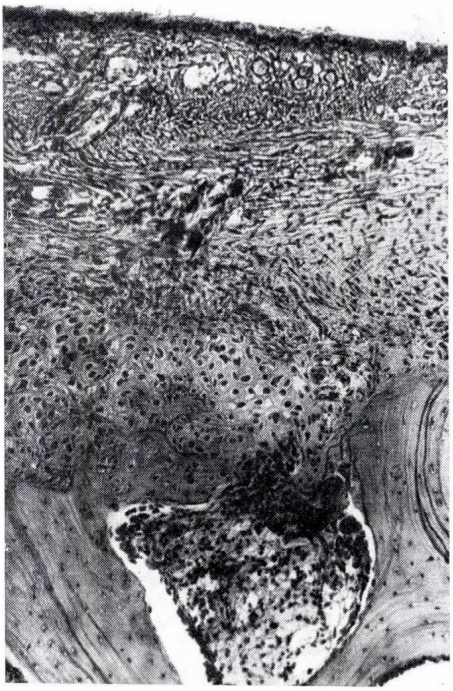

FIG. 5. Thirty-three-day-old articular surface. "Isolated cartilage islet" and its environment. Haematoxylin-eosin; ×95

distance from the cartilage islets—marked capillary network has developed (Fig. 5);

5. Seventy days after operation several cartilage islets, which were separated until then, become fused forming large confluent islets containing numerous mature cartilage cells. The tissue of the regenerating articular surface is poor in capillaries and the lumina of the vessels are, in their majority, compressed (Fig. 6).

On the results obtained on the experimental material described above, the following points will be discussed. As it has been pointed out, *cartilage tissue develops in avascular milieu.* This statement is based on concordant results of several investigations. Similar results were obtained in our investigations in which *injections* of India ink were employed (Hadházy et al. 1959) by which the capillary network of the operated articulation of the dog was visualized. In all of the cases, cartilage formation was found to be preceded by a capillary pauperization appearing first in patches, then extending over larger areas. This statement was verified by *planimetric* examinations of the cross-sections of the capillaries in the articular surface (Hadházy et al. 1959) and, by determinations of its haemoglobin content by

FIG. 6. Seventy-day-old articular surface. "Confluent cartilage islet" and its environment. Haematoxylin-eosin; ×95

chemical (Oláh et al. 1959) and chemiluminiscence (Hadházy et al. 1966) methods. Figure 7 shows the scheme of the results obtained in injection experiments.

The study of vascular conditions was followed by examinations on carbohydrate metabolism. The *oxygen consumption* (respiration) of the chondrifying granulation tissue and that of the granulation tissue adhering to the capsule, (Oláh et al. 1961) determined by the manometric method of Warburg (1926), was found to increase in the early stages and to decrease considerably in the later stages. While the ascending part of the respiration curve seemed to be explainable, according to Warburg (1926), Brock, et al. (1939), Kaunitz and Selzer (1938) and others, by the "irritation" of the metabolism due to impairment, it was rather difficult to explain the decreased respiration rates in the later stages. On the basis of the findings of Büchner (1956), examinations were performed to see whether we have to deal here with impairment of the cytochromes, deficient substrate or oxygen supply, or disturbed thyroid activity (Hadházy et al. 1961). The investigations on cytochrome and oxygen deficiency were carried out by the manometric method (Warburg), by adding methylene blue and glucose, respectively, and the thyroid was examined by histological methods. The results of our examinations indicated that the decrease in respiration is caused—in addition to the probable state of hypoxia—by reduced terminal oxidation. The examinations on aerobic and anaerobic glycolysis (Hadházy et al. 1962), performed likewise by Warburg's method, partly yielded unexpected results. In the early stages, both under aerobic and anaerobic conditions, an increase of glycolysis was observed; in the later stages, however, glycolysis was found to decrease. The early increase of glycolysis seemed to be in agreement with, and explainable by, the state of hypoxia of the tissue due to increased vascular pauperization, but the decrease of glycolysis in the later stages—on account of the stagnating or even increasing constriction of the total volume of capillaries—seemed to be inexplicable. Determinations on tissular lactic acid performed by Barker-Summerson's method gave results entirely concordant with those concerning glycolysis (Oláh et al. 1959).

Further results were obtained from our examinations on mucopolysaccha-

rides. By histochemical examinations (Hadházy and Perjés 1962) it was demonstrated that the precursors of cartilage (prechondroblasts and in particular the chondroblasts) produce large amounts of acid mucopolysaccharides. Investigations on substances containing hexosamine (mucopolysaccharides), by the method of Elson and Morgan, revealed significant accumulation of these substances simultaneously with the appearance of the confluent cartilage islets (Oláh and Hadházy 1962). The production of substances containing hexosamine in the presence of glutamine was measured both under aerobic and anaerobic conditions (Oláh et al. 1965). It was found that the regenerating articular surface is able to synthesize mucopolysaccharides both in aerobic and anaerobic milieu. This finding indicates that in the course of regeneration of the articular surface the formation of mucopolysaccharides occurs *in situ*, i.e. they are not transported from elsewhere to the chondrifying area.

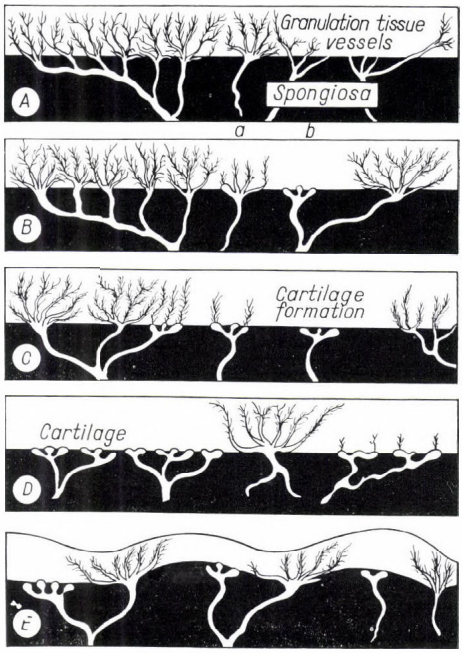

FIG. 7. Summarizing scheme of results obtained following operation. A = 7-day stage: rich capillary network in the granulation tissue; B = 20-day stage: decreased vascularization in some circumscribed areas; partial (a) and complete (b) vascular obstruction; C = 33-day stage: larger poorly vascularized and avascular areas; D = 70-day stage: small but still fairly vascularized areas within large avascular territories; E = role of mechanical factors: poorly vascularized areas corresponding to emerging spongious bone parts and fairly vascularized areas in the sunken parts of the spongiosa

On comparing our results concerning vascularization, production of lactic acid (aerobic and anaerobic glycolysis) and lactic acid content, as well as hexosamine production and content, a temporal correlation appears to exist between them. Cytochrome impairment (or inactivation) becomes manifest in a certain stage of reduction of vascularization, and the accumulation of materials containing hexosamine is parallel with the decrease in lactic acid content. This interrelation is illustrated in Figs 8 and 9. In Fig. 9 the results of determinations on haemoglobin, expressed in ferri ions, as well as on lactic acid and hexosamine are projected on each other. The cross-sections of the capillaries have been measured planimetrically and compared with the whole territory of the articular surface. The Fe^{+++} values have been determined according to Wong's method. The values represent the amount of haemoglobin.

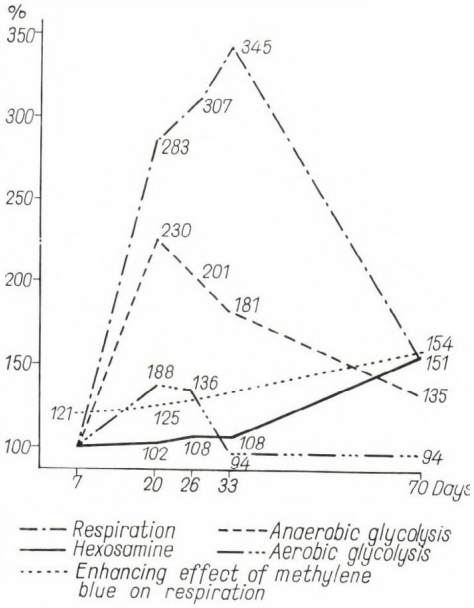

FIG. 8. Graph illustrating the alteration of respiration, anaerobic and aerobic glycolysis, hexosamine content and the enhancing effect of methylene blue on respiration. (The values obtained on the seventh day have been considered as 100%. The later values have been compared to these values)

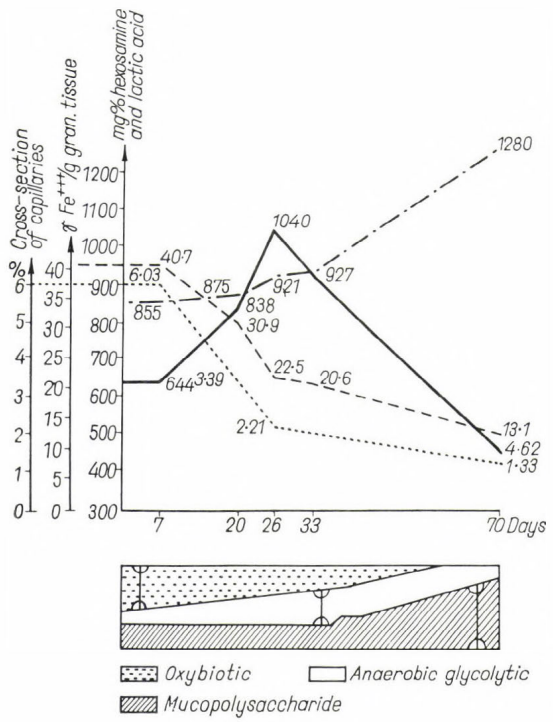

FIG. 9. Graph showing the shifting of metabolism during the development of cartilaginous articular surface from granulation tissue (7 to 70 days)

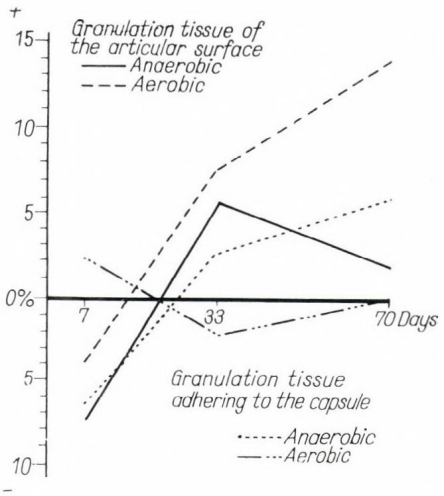

FIG. 10. Graph demonstrating the hexosamine production of the granulation tissue of the articular surface and of the granulation tissue adhering to the articular capsule under aerobic and anaerobic conditions. The amount of hexosamine produced in one hour is expressed in the percentage value of the hexosamine content of fresh tissue. After a time the chondrifying granulation tissue produces hexosamine both under aerobic and anaerobic conditions, whereas the hexosamine production of the granulation tissue adhering to the capsule occurs only in anaerobic milieu

First maximum (lower figure, left side): the conditions of oxybiosis in the initial stages are given. *Second maximum* (middle part of lower picture): the capillaries necessary for oxybiosis are reduced. Lactic acid content reaches its maximum. Cartilage islets appear. *Third maximum* (lower figure, right side): the conditions of oxybiosis (blood supply) no longer prevail. Reduction of lactic acid content indicates the decrease of anaerobic glycolysis. Mucopolysaccharide content reaches its maximum. On the basis of these data it may be supposed that the decrease of lactic acid observed in the later stages and the simultaneous decrease of glycolysis may be connected with the production of substances containing hexosamine in the following manner: some of the intermediaries of the Embden-Meyerhof pathway, as mucopolysaccharide precursors, may be used to form these substances (Hadházy et al. 1963). This seems to be supported by the obligatory hexosamine production of the chondrifying granulation tissue (Fig. 10).

The purpose of our present investigations is to verify these interrelations. Consequently, examinations are being carried out to study the changes of certain enzymes of the Embden-Meyerhof pathway taking place during chondrification. The results obtained so far indicate that the activity of phosphofructokinase enzyme has a decreasing tendency in the course of cartilage formation. It is supposed, therefore, that in cartilage formation (and presumably in all cases when and where mucopolysaccharides are formed) phosphofructokinase becomes partially blocked in the Embden-Meyerhof pathway, by which glucose-6-phosphate or fructose-6-phosphate may be transformed to hexosamine precursors. The enzymes preceding the supposed partial block appear to function normally. This statement is substantiated by our determinations on phosphohexosisomerase (Hadházy et al. 1966 and phosphoglucomutase (Oláh et al. 1966) activities.

Investigations on more distant interrelations as well as on causal motives will be the subject of further examinations.

SUMMARY

Chondrogenesis of regenerating articular surface was studied on experimental material obtained from the dog. According to previous observations, this chondrogenesis takes place in a poorly vascularized environment.

The authors have been concerned with studies of certain aspects of carbohydrate metabolism (tissue respiration, aerobic and anaerobic glycolysis, lactic acid content, hexosamine production and content, and activity of certain enzymes).

As a result of their examinations, the authors claim that previous reduction of vascularization is of utmost importance in postembryonal cartilage formation. In the authors' opinion, the increasing lack of oxygen leads to impairment of terminal oxidation or to its inactivation, by which the rate of glycolysis becomes even more preponderant in the granulation tissue. However, in the course of the Embden-Meyerhof pathway, phosphofructokinase is presumably partially blocked as a result of which some intermediaries preceding the block may be used for mucopolysaccharide formation.

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Symp. Biol. Hung., 7, pp. 65—74 (1967)

ARE THERE MEANS TO PROMOTE CALLUS FORMATION?

by

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IT HAS long been demonstrated by Krompecher, in experiments and histologically, that under compression callus develops, i.e. a bony healing ensues, while under the effect of traction only a connective tissular connection arises between the fractured ends. I wish to give here an empirical account of my experience on callus formation.

Since 1912, i.e. for 53 years I have treated more than 140,000 recent bone fractures. In the majority of the cases consolidation of the fractures occurred, though we have never administered any of those innumerable medicaments which are said to promote callus formation. *My experience has proved that no such material is available so far*, though it would be urgently needed. If sometimes no callus formation occurred, we succeeded, in the course of years, in finding out its causes and avoiding them in similar cases in a pure empirical way without making use of animal experiments or microscopes.

Apart from recent fractures, we have also treated several thousands of injured men with *delayed callus formation* and *pseudarthrosis*. All of them had previously received for a longer or shorter time, sometimes even for several years, various sorts of substances supposed to stimulate callus formation. The fact that these patients came to us with a pseudarthrosis proves that these medicines were not effective with them. In connection with a case of shin-bone fracture, I have described all that had been given 30 years ago to stimulate callus formation, in the 12/13th German edition of my work: *Technik der Knochenbruchbehandlung* (p. 262) and in my book: *Treatment of Fractures* (Fifth English edition, p. 258) and in the Spanish, Italian, French, Polish, Russian, Chinese and Hungarian editions. I have read hundreds of similar case histories. In the meantime innumerable new materials have been recommended.

In the Austrian Emergency Hospitals more than 14,000 shin-bone fractures have been treated. Of this number only 4 had pseudarthrosis. Of 1019 fractures of the shaft of the humerus also 4 cases of pseudarthrosis were observed. These 8 patients had been treated by distraction. Does the fact that the majority of the fractures healed without callus forming materials and the patients with pseudarthrosis brought to us had, without exception, received many kinds of callus promoting material, permit us to draw the conclusion that these materials were the cause of pseudarthrosis? Certainly

not as the thorough re-examination of the antecedents revealed that in these cases the three fundamental principles of fracture treatment: *reduction, immobilization* and *functional activity (exercise)*, were not properly followed trusting the effect of the callus building material given to the patients. Sometimes reduction was deficient, sometimes the duration of rest was too short and in many cases movement therapy was neglected.

Cause of callus formation. The formation of callus occurs at sites where the bone suffered such a violent impact that it broke. Thus, fracture is the prerequisite of callus formation. According to Küntscher, callus-like formation may arise in non-fractured bones as well, e.g. if rusty wires are introduced into the medullary cavities, i.e. as a result of a strong chemical irritation. The better the fragments are adjusted and the more carefully immobilized and the better their blood supply is, the sooner the callus develops and the stronger the bone becomes. Good blood supply is the most important condition of callus formation.

Delayed callus formation and causes of pseudarthrosis. Resorption of fractured ends. The fracture of a bone is soon followed by an increase of blood supply in the fracture area where lively processes of resorption and reconstruction are taking place. Both fractured ends necrotize in an area of about 1 to 2 mm, as has been demonstrated histologically (see Figs 4304 to 4307 of the Suppl. Vol. of 12/13th German edition of *Technik der Knochenbruchbehandlung.** This necrotized tissue will be absorbed and acts as a biological stimulus on the environment. Mechanically, this process is of great importance since in fractures without dislocation, a gap is formed between the broken bone ends which must be filled up. The formation and development of this gap can be fairly demonstrated in the fractures of the scaphoid. The interfragmentary space is often so narrow that it is almost indiscernible in the first roentgenogram (Fig. I/1253). Ten to 14 days later the resorption of the broken bone ends becomes obvious by the broadening of the fracture gap. If adequate immobilization is ensured, the gap is usually bridged over by bony union within six weeks. If rest is not ensured, resorption of the bone ends progresses, bringing about a traumatic cavity (Figs I/249, I/1269, and I/1270). If the bone is not immobilized even now, both fracture ends become delimited and a pseudarthrosis ensues (Figs I/1273 and I/1274) leading sometimes even to the necrosis of one or both broken bone ends (Figs I/1275 and I/1276).

Duration of consolidation. The time necessary for callus formation varies not only depending on the size of bones concerned, but also on the sort of fracture (rotation, bending, shearing, avulsion), site of fracture (diaphyseal, metaphyseal, extra-articular or intra-articular), type of fracture (direct or indirect, closed or open), and on the age and general physical condition of the injured.

* All page and figure numbers mentioned in the text refer to the 12/13th German edition of my book: *Technik der Knochenbruchbehandlung;* to the supplement volume of the 12/13th German edition; 3rd volume of 9/11th German edition of *Die Marknagelung nach Küntscher;* to the 5th English edition of my *Treatment of fractures,* or to other translations of my books.

In practice we have observed that the younger the patient and the better the blood supply of the bone segment, the more rapid callus formation is obtained. Fractures of the metaphysis usually heal more rapidly than diaphyseal fractures. In cases of closed fractures, extensive stripping of the periosteum occurs which very soon brings about a strong subperiosteal callus (Figs I/793 to 800). Comminuted fractures also heal quickly if no distraction is applied, and the broken ends are compressed to each other when required. Slow callus formation occurs in transverse fractures of the distal third of the tibia and ulna, even in those of the median thirds. Repair of a transverse fracture of the tibia requires a period of 12 to 20 weeks and that of the ulna about 10 to 12 weeks, since the medullary cavities of these bones are only slightly opened and the periosteum is torn transversally without being stripped from the bone, and all the capillaries running along the Haversian canals are ruptured. In addition, the ventral medial sides of the tibia are uncovered by muscles. Poor blood supply often manifests itself in tumours of the leg.

Moreover, in isolated fractures of the tibia, the fibula acts as a locking bone, just as the radius does in ulnar fractures. In such cases compression of the fractured bone ends following resorption of the necrotized bone surfaces is inhibited. Series of roentgenograms demonstrate that callus formation usually begins only on the lateral and dorsal side, while bone resorption proceeds on the ventral and medial sides (Figs I/294 to 301). Transverse fractures of the thigh and upper arm (Suppl. Vol. Figs 4291 to 4303) also heal rapidly, provided they are not subjected to excessive traction and the bones have a chance to shorten by some millimetres, since they are surrounded on all sides by muscles.

Primary bone healing. As has been shown by Schenk and Willenegger, fractures of the shaft may likewise heal without visible calluses, and the vessels of one fragment can grow into the other without resorption of the fracture surfaces and visible callus formation, just as we have long ago observed in fractures of the scaphoid and those of the femoral neck.

General causes of delayed callus formation and pseudarthrosis. Tuberculosis, osteomalacia, rickets, etc. can have a delaying effect on callus formation, though not inhibiting it altogether.

Influence of hunger, cold and epidemics on callus formation. We have often heard and read that callus formation can be delayed or inhibited by starvation, malnutrition, vitamin deficiency, cold and infectious diseases. This, in fact, occurs if these unfavourable circumstances last for months. Influences of shorter duration have no effect whatever on callus formation, as has been demonstrated in the following mass experiment. In the winter and early spring of 1944, German troops had to retire from Russia under the most adverse circumstances. The wounded soldiers were lying in cold melting snow, badly splinted or not put in splints at all, until they were carried to the nearest dressing station. Sometimes it took several days to get to a collecting station where usually good transporting plaster casts were applied. So they arrived to Vienna after a long journey, frequently at the end of the third or fourth week. Their food supply was often insufficient and many of them suffered of diarrhoea. In spite of all this, in the ma-

jority of the cases the roentgenograms taken after three weeks displayed good callus formation (Suppl. Vol. Fig. 4316). These fractures healed by shortening as they had not been subjected to distraction.

In the previous years an exceptionally great number of patients with greatly delayed callus formation or pseudarthrosis were brought to our hospital (Suppl. Vol. Fig. 4317, and *Die Marknagelung nach Küntscher,* Figs 3351 to 3419), who had been treated in well-equipped hospitals by prolonged traction applying heavy weights, or by small, improperly applied plaster casts on the chest and upper arm (Fig. I/734h). These patients had been lying in good beds, had been well nursed and well nourished and they did not suffer of cold. Yet, their callus formation was not satisfactory because in most of the cases the bone fragments were distracted bringing about a diastasis between the fragments.

These results observed in ten thousands of fractures seem to furnish clear evidence that rapid callus formation may occur even under unfavourable circumstances, if fracture healing takes place by shortening of the broken bone and no callus formation occurs if distraction is applied by which a diastasis arises between the fractured ends.

Local disturbances in the fracture site usually bring about disturbed callus formation and pseudarthrosis. We shall not discuss here the local diseases of the bone (metastases of carcinoma, sarcoma, gummae, osteomyelitis, etc.), but only the local disturbances occurring in healthy bone. These are most frequently due to deficient or inadequate treatment.

The main causes of delayed callus formation and pseudarthrosis may be enumerated as follows:

1. Omission of manipulative reduction or immobilization of too short duration in intra-articular fractures with displacement, as in various fractures of the femoral neck and of the scaphoid;
2. Dislocation of the fragments as a result of accidents, e.g. in fractures of the patella, fibular head, internal ankle, olecranon, epicondylus lateralis, various apophyses (proc. coronoideus, proc. spinosus, proc. transversus etc.). Displacements with lengthening of the bone are rarely encountered in shaft fractures ([II]2 2434);
3. Inhibition of pressure of the fragments to each other in fractures of the forearm or leg when only one bone is broken and the other acts as a locking bone;
4. Distraction of the fragments with a large gap between the fractured ends owing to excessive traction;
5. Distraction of the fragments owing to technically deficient plaster cast with double wires or double pins (transfixion);
6. Distraction of the fragments due to tennis racket bandage *(Die Marknagelung nach Küntscher,* Figs 3424 and 3425);
7. Removal of callus-building materials and inhibition of impaction of the fragments into each other in certain kinds of osteosyntheses;
8. Interposition of periosteum between the fragments, e.g. in fractures of the inner ankle or fracture dislocation of the scaphoid;

9. Massage and passive movements applied immediately after injury in intra-articular and in shaft fractures;
10. Immobilization of too short duration or frequently suspended immobilization by repeated attempts of reduction;
11. Comminution of open fractures;
12. Infection of fracture sites.

1. Synovial fluid was previously believed to inhibit bony unification in pure *intra-articular fractures* as in those of the *femoral neck* or *scaphoid*, in which both broken ends penetrate freely in the joint, as well as in fractures where only the fracture surfaces enter the joint without lying freely in it, e.g. with fractures of the patella, olecranon or fracture dislocations of the inner ankle. In unimpacted fractures of the femoral neck and those of the scaphoid, usually pseudarthrosis arises as a result of formerly employed treatment of massage and passive movements (Figs I/1261 to 1276 and II/1 1814 to 1869). In such cases the blood supply of the fragments is impaired, the periosteum is thin and they are uncovered by muscles. Therefore, callus formation can only occur if the new vessels from the spongious medullary cavities of one of the fragments grow into the other fragment (Figs I/302 to 311, *Primäre Knochenheilung*). The slightest side movements of the fragments against each other must shear and destroy these newly formed vessels and this, if no adequate immobilization is ensured, will lead to further progress of resorption and absence of bony unification. Pseudarthrosis encountered in such fractures and necrosis ensuing in fractures of the scaphoid can be avoided if careful manipulative reduction of the fracture is followed by *uninterrupted* immobilization for a prolonged time. In such fractures blood supply is bad, the fragments being poorly vascularized. Therefore, immobilization must be continued for a long time, namely in fractures of the medial femoral neck for 6 to 8 months, and in fractures of the scaphoid for about 6 to 10 weeks, and sometimes even longer immobilization is required.

As to the synovial fluid being not responsible for inhibited callus formation, the evidence obtained from 130 cases in which follow-up examinations were performed, seems to be convincing. Of 130 cases of recently operated femoral-neck fractures (Ender 1952), 121 (93%) and of 556 re-examined recent fractures of the scaphoid (Böhler et al. 1954), 557 (96·5%) healed by bony unification.

In ankle fractures in which the fractured ends always penetrate into the spring-joint where they are washed by the synovial fluid, bony healing is usually obtained, provided there is no interposition of periosteum.

Fractures of the radius in which the fractured ends often penetrate into the hand joint always show bony healing just as the majority of other joint fractures.

2. Fractures of the patella, olecranon, fibular head and median epicondyle always heal by bony unification, provided careful manipulative reduction and adequate osteosynthesis (possibly by wire suture or transfixion) has been performed. The extent of diastasis in patellar and olecranon fractures marks the degree of injury of the extensor apparatus.

3. In fractures of the leg where only the shin bone has broken, delayed callus formation may occur (II/2 Figs 2439 to 2442). By osteotomy of the fibula the broken bone ends can be apposed and pressed to each other by which a rapid bony healing ensues (II/2 Figs 2443 to 2446).

4. *The most frequent cause of delayed callus formation and pseudarthrosis is for the present the application of too heavy weights in prolonged traction treatment. The farther the fragments are distracted and the longer the duration of traction, the more severe and lasting are the impairments due to excessive traction.*

5. Transfixion in which two wires or two pins are used in addition to plaster bandage, may cause delayed callus formation and pseudarthrosis if prior to the application of plaster the fragments were too strongly distracted.

6. Excessive distraction of the fragments in tennis racket bandage also leads to pseudarthrosis and dystrophy.

7. In shaft fractures, especially in transverse fractures of the tibia and fibula, osteosynthesis is often followed by pseudarthrosis because in such cases the haematoma and other callus-building materials have been removed from between the fractured ends and because with many operative treatments, e.g. with employment of plates with screws without compression or when strong wire sutures are applied, the fragments are prevented from pressing to each other which is necessary for healing. By resorption of the fractured ends a gap arises between the fragments (I/1051 to 1054). The medullary nail often exerts a locking effect *(Die Marknagelung nach Küntscher,* Figs 3466 to 3475). Oxidizing metal may cause considerable destruction of the bone, leading to pseudarthrosis (II/1 Figs 1798 to 1801). The danger of pseudarthrosis can be avoided if fine bone grafts are used at the operation (Böhler 1950, 1955, Böhler and Rupp 1952).

8. Interposition of pieces of periosteum in ankle fractures (II/2 Figs 2710 and 2711) can be easily recognized by the imperfect apposition of the inner ankle, though the spring bone has been adequately reduced. Here pseudarthrosis can only be prevented if the fragments are freed, the interposed periosteum is removed and adequate fixation of the ankle is ensured by transfixion (II/2 Figs 2707 to 2716). If in dislocation fractures the fragments of the scaphoid do not lie in perfect apposition and the resultant large gap persists between the broken ends, the fracture site must be exposed and the fragments of periosteum and capsule removed. Finally, immobilization must be performed with a fixing wire (I/Fig. 1228) or traction screw. Pseudarthrosis as a result of muscle interposition is rarely encountered and usually only in cases if excessive traction has been applied *(Die Marknagelung nach Küntscher,* Figs 3355 and 3356).

9. Massage and passive movements practised immediately after injury, as was frequently done formerly, often result in delayed callus formation and pseudarthrosis, especially in fractures of the scaphoid and femoral neck. Fortunately, this treatment is nowadays very rarely employed.

10. The duration of immobilization is often too short due to premature removal of the fixing bandages so that the joint might be actively exercised. The movements, however, occur at the fracture site until no sound union

has taken place. In fractures near joints which are surrounded by short fibred muscles liable to myositis ossificans (subtrochanteric- and pretrochanteric femoral fractures and fractures of the elbow), too short immobilization with treatment of massage and passive movements inevitably leads to abundant callus formation, whereas adequate reduction and uninterrupted immobilization of the broken fragments until sound union has been achieved will always result in calluses of normal size. In inadequately immobilized lateral fractures of the femoral neck, abundant callus formation starting from the periphery of the fragments and pseudarthrosis initiating from the centre may be observed side by side.

11. Comminution of open fractures results in defect pseudarthrosis. If in fractures caused by forced bending a wedge-shaped bone fragment has broken off which is then removed, no bony healing is achieved. In World War I many fractures caused by bullets were comminuted. French surgeons adopted in those times a very radical method of removing all bone splinters in most bullet wounds and joint injuries. According to a statistical account, of 37,745 femoral fractures due to bullet wounds amputation had to be performed in 10,908 cases (28·9%) on account of pseudarthrosis developed in the fracture sites.

12. Delayed callus formation and pseudarthrosis resulting from infection can be avoided if the wound is freely excised and skin closure is performed without application of vascular ligatures and deep sutures, and if after operative reduction an unpadded plaster bandage is applied which must be immediately cut along to the last thread of gauze. If the wound becomes infected after all, adequate draining must be ensured, otherwise the bone splinters will be bathed in pus and necrotize. The periosteum will be detached from the bone.

If the sequestrated bone lying between the fragments is removed in time, rapid bony healing frequently ensues. Excessive traction is particularly damaging since it may bring about recurrence of infection *(Die Marknagelung nach Küntscher*, Figs. 3772 to 3383).

Delay and inhibition of callus formation due to vascular spasms. If by prolonged powerful traction or imperfect osteosynthesis distraction of the fragments occurs, compression of the fracture ends being inhibited, vascular spasms arises by which blood supply is impaired. In case of excessive traction of long duration not only local disturbances but also severe vasomotoric impairments occur leading to dystrophy of the whole limb (Sudeck). The signs of severe vascular disturbances may be enumerated as follows.

Rapid decrease of the calcium content of bone causing pains (Fig. 4314). This is followed by: *shrinkage*, *cornification* and *glistening* of the skin. The *hands and legs involved become smaller* even with adults (Fig. 4315). The limb becomes *cyanotic* and *cold. Tendency to swelling* is frequent. *Extensive atrophy* of muscles occurs. Especially the small muscles of the foot become rapidly atrophied and shortened by which clawing of the toes develops (Fig. 4315).

Shrinkage of ligaments with subsequent stiffness of the joints. The picture is similar to that seen resulting from milder forms of acute ischaemia.

Grossly excessive traction can frequently result in paralysis of peroneal and ischiatic nerves *(Die Marknagelung nach Küntscher,* Figs 3372 to 3383).

In *open fractures,* inaddition to severely impaired blood flow, the disturbance caused in the tissue by excessive traction of the fragments may cause various complications: *strong inflammation* and subsequent *increased purulation, gravitation abscesses* and *pushing out of fragments of sequestrated bone (Die Marknagelung nach Küntscher,* Fig. 3374) which were already in the course of healing. Often *coronary sequesters* are seen at both broken ends (Fig. 4317).

It is of particular importance, therefore, to be careful in applying prolonged traction to old, open or infected fractures.

If the weights employed during traction treatment are only a little too heavy and the duration of traction is not too long, only a delayed callus formation will result without other harmful effects.

The impairment of bone, especially pseudarthrosis, can often be avoided by adequate surgical intervention, but severe nutritional disturbances are usually irreversible.

In investigating the causes of pseudarthrosis in cases where no primary defects are concerned, it is usually found that in such cases compression and necessary shortening of the fragments have been inhibited during treatment.

Experimental investigation on callus-promoting substances. It would be most fortunate to find a material which would promote callus formation. No such substances have been found so far, though many have been recommended to us.

In experiments on the effect of such substances it should be observed whether, before or during administration of the substance in question, the bone fragments were shortened by compression or lengthened by distraction and whether young or old experimental animals were used. It is known that the younger a bone, the more rapidly it consolidates. On studying large numbers of roentgenograms of experimental animals and of human beings to demonstrate the effect of certain substances promoting callus formation, I have often observed persistent epiphyseal cartilage evidencing young age. So rapid callus formation ensued not as a result of callus-forming material, but owing to young age. As we know, callus formation always occurs if the bone had been exposed to a violent impact causing fracture. If bone is lengthened by traction or a diastasis is produced, no callus formation ensues. On the other hand, if shortening about 1 mm is permitted or the broken ends are compressed, rapid callus formation occurs. In the majority of fractures callus formation can be evidenced roentgenologically, even three weeks following injury (Fig. 4216). It is not the new callus-forming material that brings about rapid callus formation but shortening of the broken fragments.

From the foregoing it may be stated that *pseudarthroses, in the majority of the cases, are avoidable consequences of treatment and do not inevitably follow from accidental injury.*

The question is how can we prevent failure of callus formation and pseudarthrosis. This can be achieved if we avoid the causes enumerated

above, in particular the distraction of the fragments whether by grossly excessive traction or inadequately performed osteosynthesis.

Since the incidence of pseudarthrosis is still rather high, teaching and organization of traumatic surgery should be further improved.

What attitude should the practical surgeon adopt with respect to callus-promoting material? Since these materials, according to my experience, are ineffective, they should not be prescribed for the patients. I have often asked my patients which of the numerous callus-forming materials do they wish to take. When I informed them of my experience that I have never seen any good effect of such materials on fracture healing but very often stomach disturbance was the only result obtained, they renounced to take them. We hope that the proceedings of the next few days when the newest results of researches will be reported, we shall get nearer to our aim, i.e. to find a material that would indeed promote callus formation and would accelerate the healing of fractures.

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Symp. Biol. Hung., 7, pp. 75—86 (1967)

MORPHOLOGICAL FINDINGS IN PRIMARY FRACTURE HEALING

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THE CLINICAL and radiological observations made in connection with the healing of fractures treated according to the principle of stable osteosynthesis have raised a great many new basic-research problems (see details by Segmüller 1966). Certainly the most intriguing of these is the problem of so-called *primary fracture healing*. This process, which to all intents and purposes consists in healing without radiological evidence of callus formation, was propagated by Danis (1949) under the name '*soudure autogene*' (autogenous weld) as being the ideal mode of healing in shaft fractures. If we have opted for the term 'primary fracture healing', then we have done so simply by analogy with the accepted expression 'healing *per primam intentionem*', which is used in surgery and pathological anatomy. The main criterion in this form of healing is the absence of scar formation. Scarring may be described as the replacement of a defect or the restoration of continuity within highly differentiated tissue by a more primitive form of tissue. Fundamentally, however, callus is no more and no less than a type of scar. These considerations prompted Lane, as long ago as 1914, to speak of healing *per primam intentionem* when fragments were re-united directly by an osseous bond. On the other hand, osteosynthesis involving the formation of a callus of connective tissue or cartilage such as is generally found in fractures treated by conservative immobilisation methods is regarded as healing *per secundam intentionem*. While observations relating to the histomorphological processes associated with secondary fracture healing were abundant, the histological pattern of primary bone healing still remained more or less obscure. Consequently, the elucidation of these processes became a matter of considerable urgency.

Investigations and hypotheses stressing the importance of a connection between mechanical stress and the differentiation of sustentacular tissue have made a decisive contribution towards the understanding of primary and secondary fracture healing. In 1937, in his book on osteogenesis, Krompecher analysed the processes of foetal ossification in relation to local mechanical conditions and coined the phrase 'primary angiogenic ossification,' the full implications of which were not recognized until many years later. This process, which probably represents the most rational form of ossification, occurs only in the absence of mechanical stress. If the cells capable of forming bone are subjected to tensile or compressive stress, they differentiate instead to

form connective-tissue fibres and cartilage. Under the protection of this intermediate sustentacular tissue the deposition of osseous substance can proceed by way of desmal and endochondral ossification. Primary angiogenic ossification, however, involves yet another, no less significant characteristic, namely that osteoblasts can function only in the presence of an adequate capillary network. These highly differentiated cells can maintain the energetically selective metabolism necessary for the formation of bone matrix and exert an influence on the process of mineralization only if they are in direct contact with capillary blood vessels. The implications with regard to fracture healing were recognized and studied experimentally by Krompecher: he found that flexibly fixed fractures exposed to tensile or compressive stresses consolidated with a fibrous or cartilaginous callus; yet the degree of stability which could be achieved with the aid of a *fixateur externe* (external fixation clamp) proved inadequate to guarantee a primary bone union. On the basis of similar experiments, many other authors later arrived at the conclusion that a primary bone union between the fragments could be achieved provided the fixation of the fracture was sufficiently rigid (Altmann 1950; Hasche-Klünder and Gelbke 1952; Matzen 1952, 1954; Oberdahlhoff 1948). As operative techniques improved, it became more and more apparent that the extent of callus formation was dictated by the stability of the fixation; the more complete the immobilization, the less callus developed (Yamagishi and Yoshimura 1955). Friedenberg and French (1952), Bagby and Hanes (1958), Petrokov (1962) and others successfully demonstrated that fragments subjected to compression were more rapidly reunited by an osseus bond—a fact which can certainly be explained, in retrospect, in terms of improved adaptation and fixation techniques. However, the high degree of sensitivity with which the blastema capable of giving rise to osseous regeneration reacts to mechanical stresses did not become apparent until experiments with the object of producing primary healing of a shaft fracture without radiological evidence of callus formation were undertaken.

Preliminary trials in sheep (Willenegger et al. 1962) at first did not yield the desired results, because these animals—in common with rabbits and some other mammals—differ from man as regards the structure and development of their cortical bone. Experiments in the dog, on the other hand, promised to be more successful.*

The standard experiment, shown diagrammatically in Fig. 1, was finally devised to suit our requirements. For technical reasons the radius was chosen as the site. The only way to ensure that the conditions would be reproducible was to base the experiments on a transverse osteotomy performed with a fine saw; otherwise, the operative procedure corresponds exactly the method of internal fixation with the aid of compression plates practised in man. The use of a specially designed compression device permitted the compressive force exerted on the fragments to be measured with a reasonable degree of accuracy. The anatomical shape of the radius

* These experiments were supported by the Fritz Hoffmann-La Roche Foundation for the Promotion of Scientific Group Studies in Switzerland.

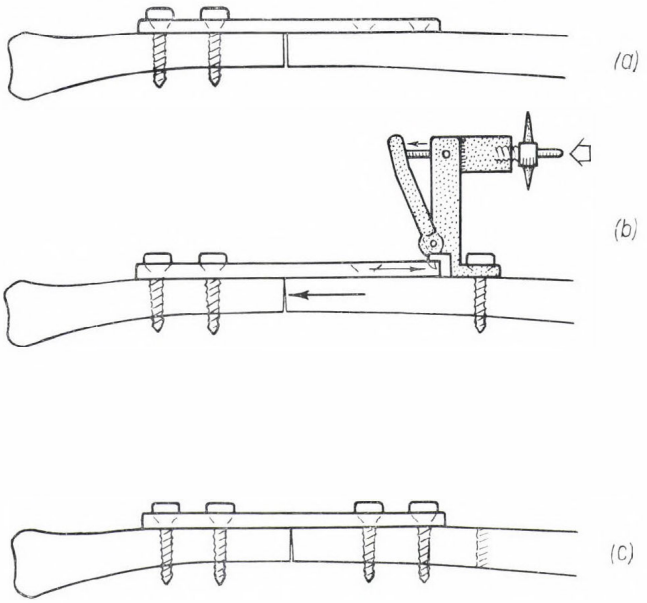


FIG. 1. Diagram showing the operative procedure adopted in the standardized experiment on the dog radius. (a) Screwing of the plate to the distal fragment (saw osteotomy); (b) Attachment of the compression device, reduction of the fracture, and compression; (c) Final fixation; compression device removed

in the dog proved to be another factor of considerable significance for the interpretation of the findings; the convex curvature on the dorsal aspect is virtually eliminated when the plate is screwed in position and the fracture gap consequently assumes a slightly cuneiform shape. The pressure required to compress the fragments is, thus, concentrated entirely on the cortical bone directly under the plate. The decisive findings relating to one instance of primary healing in the dog radius are summarized in Fig. 2: radiological signs of periosteal callus formation can be seen only along the margins of the plate and around the ends of the screws. The demarcation line between the two fragments, which was initially clearly visible, disappears within 5–8 weeks. A longitudinal section of the area shown in the X-ray photographs (Fig. 2) reveals marked endosteal and slight periosteal callus formation around the thread of the screw at the right-hand edge of the preparation; the screw is, as it were 'embedded in bone' (Wagner 1963). The osteotomy gap, on the other hand, has been replaced by bone, and no significant thickening of the cortex as a result of periosteal and endosteal bone deposition has taken place. Further magnification reveals the differences in the histological healing pattern due to the wedge-shaped deformation of the fracture gap. Directly below the plate (Fig. 2c) the ends of the fragments have been tightly compressed and the osteotomy gap has been reduced to an extremely fine line, into which neither cells nor blood vessels can penetrate. Consolidation at these points takes place by way of very slight endosteal and periosteal deposition and—a point to which we attach great significance—the regeneration of Haversian systems, which

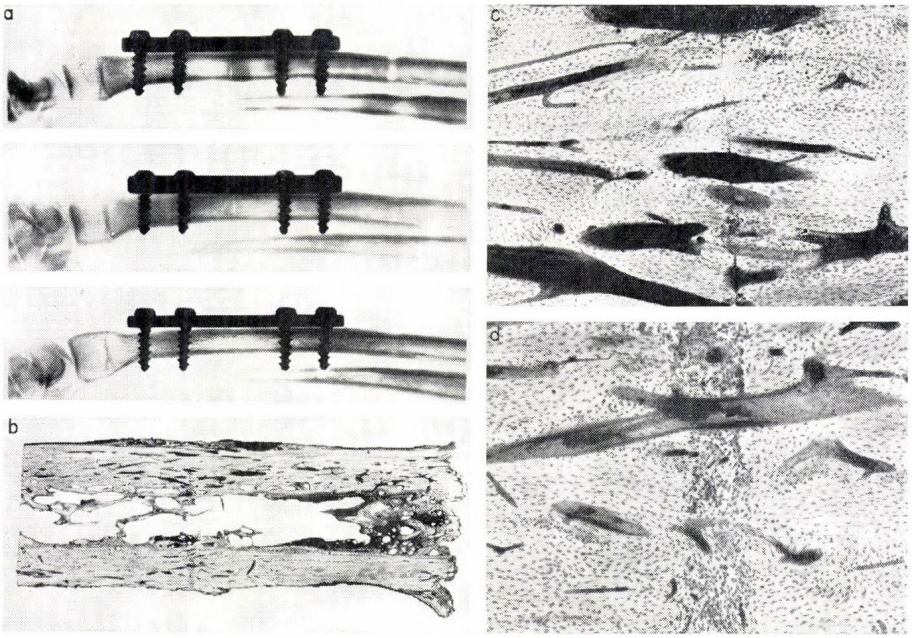


FIG. 2. Primary healing after transverse osteotomy of the dog radius. Non-decalcified longitudinal section stained with fuchsine (Krompecher-Frost). (a) X-ray photographs taken after the operation and 5 and 8 weeks later, by which time the gap has closed without visible evidence of callus formation; (b) Lens magnification of a longitudinal section through the same radius. Massive ossification around the thread of the screw visible on the right. Closure of the osteotomy gap with only slight periosteal and endosteal involvement; (c) Section from the cortex immediately below the plate. The fragments are in direct contact and only a fine vertical line betrays the presence of the osteotomy gap. Darkly stained regenerating osteons that have grown across from one fragment into the other can be seen; (d) On the opposite side the operation left a slightly wider gap (cf. X-ray photograph and Fig. 1). This was closed by primary regenerated tissue and penetrated by regenerated osteons

grow across the gap from one fragment into the other. On the side opposite the plate the ends of the fragments remained separated by a narrow gap after the operation (Fig. 2d). This is first filled by bone; the regenerated tissue, however, still displays an unorganized structure. Not until the gap has been closed in this way can regenerating osteons restore the original structure of the cortical bone and, thus, finally consolidate the bond between the two fragments.

In a series of dogs sacrificed at intervals of one week we have successfully demonstrated the chronological order of the stages of these processes (Schenk and Willenegger 1963, 1964, 1965). Briefly, it may be said that blood vessels and accompanying osteoblasts enter the open fracture gap already during the first week. Provided the fixation is absolutely stable, they begin to deposit fresh bone on the exposed ends of the fragments and, thus, repair the defect. To fill this gap, which is 1 mm at the widest, 2–3

weeks are required. Roughly at the same time active reconstruction marked by greatly intensified regeneration of the Haversian systems begins within the cortical bone in the vicinity of the site of osteotomy. It has long been known that osteons constantly undergo a process of regeneration. With the aid of microradiography, autoradiography and above all the technique of tetracycline labelling, various authors have calculated the intensity of this process. The figures of Amprino and Marotti (1964) as well as of Marotti (1961) for the bones of the extremities in the dog deserve special mention; these authors found that the rate of physiological regeneration in the radius amounted to between 2% and 5% of the total number of osteons. All these investigations are based on transverse fractures. How rewarding the investigation of osteon regeneration in longitudinal sections would be was not fully realized until studies of fracture healing were undertaken. We believe that this mode of regeneration is the most characteristic feature of primary fracture healing. If cortical bone does, in fact, possess regenerative powers, these can only be implemented by way of osteon regeneration, which in turn functions only in the presence of an adequate capillary network. Fracture or osteotomy inevitably involves the severing of blood vessels and the development of haematomas and localized thromboses. Within a circumscribed zone around the ends of the fragments, the size of which depends on a number of secondary factors, the osteocytes in the walls of osteons affected by the circulatory disorder die. This does not mean, however, that the bone substance is destroyed; it can fulfill its static function perfectly, even in the devitalized state. The destruction of the cells initiates a process of regeneration, the active elements of which are the surviving vessels and mesenchymal cells in the Haversian canals. Since the Haversian vessels are supplied by the periosteal and endosteal arteries via numerous anastomoses, the state of the vascular network in the medullary space and in the regions around the bone plays an important part. Haversian regeneration invariably begins with resorption. Groups of osteoclasts unite to form a kind of 'cutter-head', which moves forward through the cortex boring out a resorption canal (Fig. 3a). The route taken by these advancing osteoclasts is apparently determined by the original structure of the cortical bone. They do not necessarily make their way along deserted Haversian canals, and frequently branch off at acute angles. The possibility that internal stress in the compact bone substance influences the course of these resorption canals cannot be excluded. Up to the present, however, this question has not been clarified experimentally. Nevertheless, it is this resorption that finally determines both the direction and the diameter of the regenerated osteon.

The osteoclasts are followed directly by blastema rich in cells, which forms a kind of sleeve around centrally situated blood vessels. These mesenchymal cells give rise to generations of osteoblasts, which line the walls of the resorption canal with an epithelium-like layer (Fig. 3b). They begin to deposit new bone lamellae, which reduce concentrically the lumen of the canal. The formation of a new osteon is estimated to take 5–6 weeks in the dog and roughly 3 months in man. During this time, however, the osteoblastic 'cutter-head' is steadily pushing forwards. Under favourable

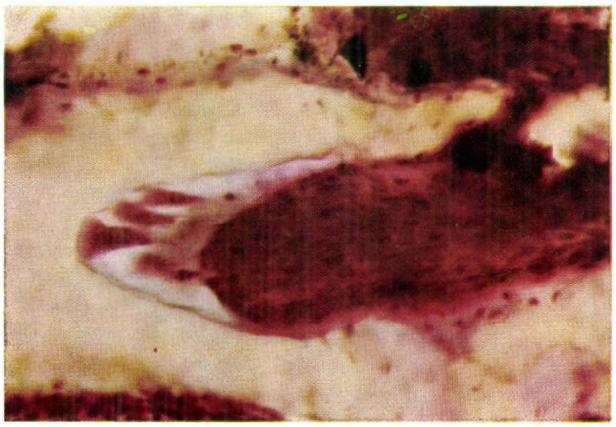
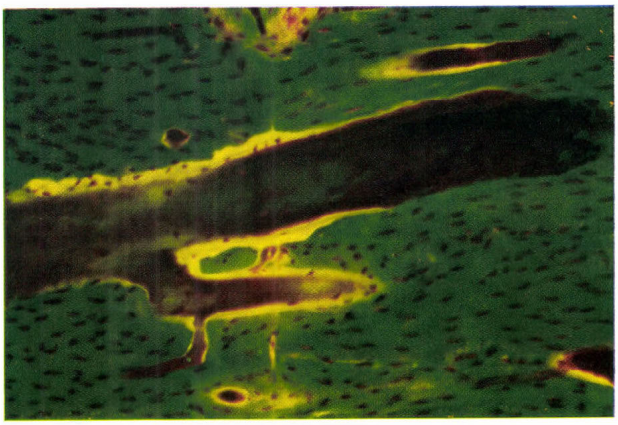


FIG. 3. Longitudinal regeneration of osteons as seen in non-decalcified longitudinal and transverse sections: (a) Fuchsine staining. Osteoclasts at closed end with osteoblasts and their precursors behind; (b) Thin section, 5 μ. Goldner staining. Osteoclasts, osteoblasts and central blood vessels. This stain makes the osteoid appear reddish brown, the calcified bone matrix, green;

conditions the rate of progress can be determined. This is made possible by the technique of tetracycline labelling, an original example of which can be seen in Fig. 3c. Since its discovery by Milch, Rall and Tobie in 1947, this technique has come to play an indispensable part in the study of bone reconstruction. The underlying principle is that tetracycline is stored in those parts of the bone substance that are taking part in the process of mineralization at the time of administration. When the date of administration is known, the rate of bone regeneration can be determined. The regenerating osteon shown in Fig. 3c was labelled just when it was about to grow through an ideally adapted section of the osteotomy. When labelling is repeated at fixed intervals the rate of longitudinal progress of the osteon (Fig. 3d) can be calculated. We have studied a large number of osteons in this respect and have come to the conclusion that an osteoblastic 'cutter-head' moves forward at a rate of 50–80 μ in 24 hours. In this way it is



(c) Osteon after traversing an optimally adapted osteotomy. Tetracycline labelling one week prior to removal of the material causes lamellar systems then in the process of calcification to appear yellow. (d) Longitudinal view of regenerating osteon following triple labelling with tetracycline at intervals of 7 days. The traces are superimposed like the sections of a telescope. The rate at which the osteon advances can be calculated from the distances between the leading edges of these traces (cf. text)

possible to show how fragments are reunited when their surfaces, following reduction and fixation, are in direct contact (Fig. 2c). The regenerating osteons bore their way like wood-worms from one fragment into the other and unite the two pieces with a sort of mortise-joint. The advantage of this mode of healing is that the restoration of the original structure of the cortex begins at once. Subsequent reconstruction such as constitutes the final phase in classical fracture healing becomes unnecessary. Of course, the stability of such a bond depends largely on how many osteons take part in this process of regeneration within a reasonable period of time. Our experiments in the dog radius yielded valuable information in also this respect. In this case our calculations have been based not on longitudinal sections but on transverse sections taken from the osteotomy at various intervals. Here again tetracycline labelling affords the assessing of the Haversian regeneration quantitatively. Achromycin was administered to the operated animals at intervals of 14 days. The osteons taking part in

the process of mineralization during these periods were thus marked once, twice, or three times. Within 8 weeks of the osteotomy about two thirds of the osteons underwent regeneration. Reconstruction begins during the third week after the osteotomy, reaches its maximum intensity between the 4th and 6th week and thereafter gradually subsides. However, it may be assumed that adequate stability will be achieved as soon as 2 months after the operation.

The principle features of primary bone healing may be summarized as follows:

1. If fixation is rigid, primary bone union takes place in interfragmental gaps;
2. Resorption of the fragment ends leading to widening of the interfragmental gap either does not occur or does so only to a negligible extent;
3. The inevitable devitalization of the fragments gives rise to intensive regeneration of the Haversian systems. The resorption canals extend along the longitudinal axis of the cortex and finally pass through the line of fracture;
4. At points where the fragment ends are in direct contact, consolidation takes place almost exclusively by way of regenerating Haversian systems.

MORPHOLOGICAL FINDINGS IN HUMANS

Our experimental investigations have also served to confirm another clinical finding, namely that the operative treatment of fractures makes colossal demands on the surgeon. Primary bone healing can only be achieved as the result of a biomechanically well-conceived plan of treatment, which is carried out with scrupulous attention to asepsis, the utmost care in dealing with surrounding tissues, and the necessary degree of technical understanding and dexterity. Even the slightest error or compromise can jeopardise the success of the operation. Failures were inevitable even in our experiments in the dog. Some of them illustrate vividly the histological consequences of typical complications. The greatest risk involved in the open treatment of fractures remains infection. We were surprised to discover radiological and histological evidence of inflammatory reactions even in free-moving animals which apparently behaved in a completely normal way and in which there were no perceptible signs of complications. Apart from leukocytic infiltration, the most impressive feature observed in these cases was an extraordinary increase in osteoclastic activity (Fig. 4). Resorption mainly involves the devitalized ends of the fragments and is marked by the widening of the fracture gap on the X-ray photograph (Fig. 4a). However, this finding can rapidly be obscured by pronounced callus formation (cf. X-ray photographs taken after 2 and 3 weeks). While the osteoclastic break-down of fragment ends is in progress, a genuine inflammatory callus consisting of a compact, excessively thick deposit of fibrous

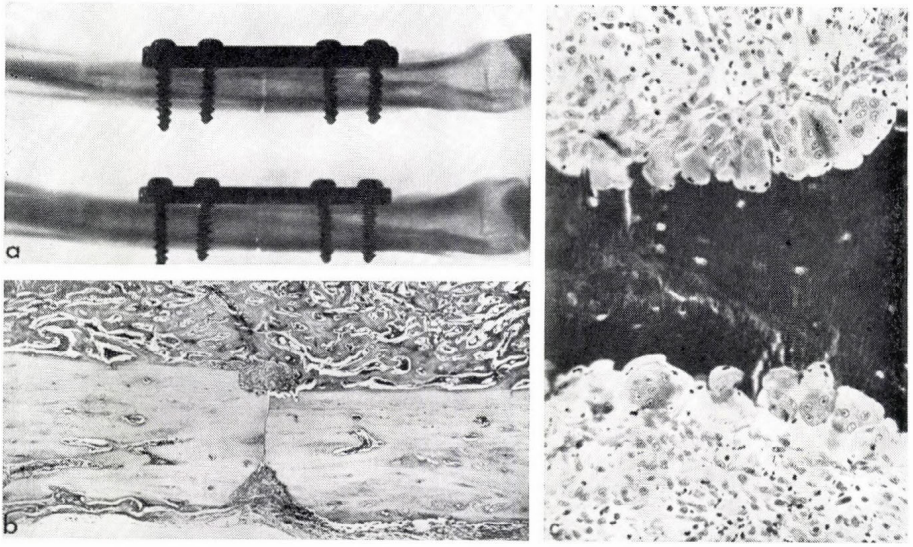


Fig. 4. Radiological and histological findings in a slightly infected osteotomy in the dog radius. (a) X-ray photographs taken after 2 and 3 weeks. After 2 weeks the osteotomy gap appears to have widened; after 3 weeks this can be seen less clearly owing to pronounced callus formation in the medullary space; (b) Striking histological features after 3 weeks are the osteoclastic resorption of the well-adapted fragment ends and the very extensive callus formation in the medullary space. Reconstruction of the cortex is just beginning; (c) Further magnification reveals the activity of the osteoclasts along a residual section of cortical bone, leading to rapid resorption of the devitalized fragment ends

bone forms in the medullary space (Fig. 4b). Processes of resorption similar to those evident in the vicinity of the osteotomy take place around the thread of the screw. The partial loss of stability can be demonstrated clearly when the plate is exposed and the screw removed. This persists even after the inflammatory reaction has subsided, so that the mechanical result is comparable to that achieved in a conservatively treated fracture. The granulation tissue penetrating into the widened fracture gap is exposed to mechanical stress which prevents it from differentiating to osseous tissue. Fig. 5a shows the radiological course of healing of an osteotomy that was accompanied by a clinically bland infection. Loosening and displacement of the screw can be seen, especially on the proximal sides, and, in addition, the dorsal aspect of the radius displays an increased degree of convex curvature. Histologically, despite extensive callus formation, the osteotomy has not consolidated after 8 weeks (Fig. 5b). The fracture gap visible on the X-ray photograph is almost exclusively filled by fibrocartilage. Periosteal and endosteal callus formation is very intensive, as is the Haversian reconstruction within the compact bone. The fibrocartilaginous disc in the fracture zone has prevented the osseus reunion of the fragments, except at a few points. Some time passes before this cartilaginous

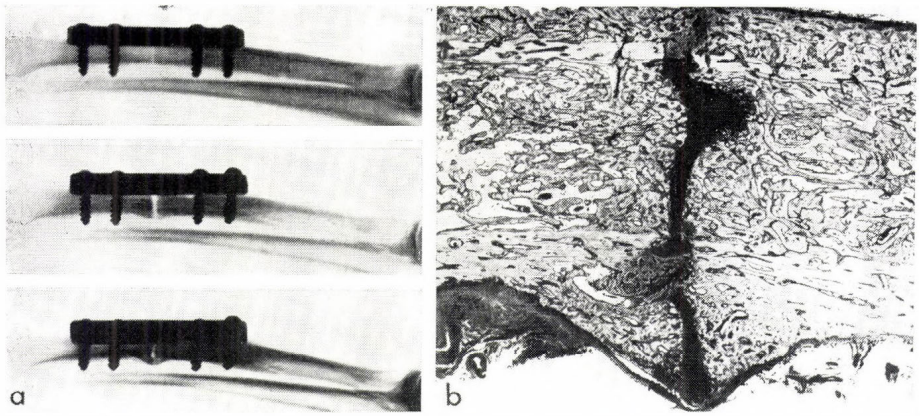


FIG. 5. Healing of a surgically treated osteotomy in which an intercurrent infection gave rise to loss of stability. (a) X-ray photographs taken after 18 days, 5 and 8 weeks. Note how the fracture gap is beginning to widen, and the loosening and displacement of the screws in the proximal portion of the bone. In addition to the endosteal callus, clear-cut periosteal callus has developed and is in the process of bridging the fracture gap, which is filled by fibrocartilage; (b) General view of an axial longitudinal section embedded in celloidin and stained with Delafield's haematoxylin. Notable features are the callus formation and the intensive reconstruction going on within the compact bone. The interfragmental fibrocartilage is darkly stained and is gradually being replaced by fibrous bone as a result of endochondral ossification

mass is resorbed as a result of endochondral ossification and is replaced by trabeculae. Not until this has taken place can functional reconstruction of the cortex and the break-down of periosteal and endosteal callus begin.

These findings invite comparative assessment of primary and secondary fracture healing. In all fairness, however, it must be emphasized that all the cases described involved surgically treated fractures. From the histological point of view, the economical pattern of primary fracture healing, leading as it does directly to the restoration of the anatomical and functional structure, is most impressive. Secondary healing, on the other hand proceeds in a more roundabout fashion, more or less highly differentiated sustentacular tissues being formed and then replaced step by step. Which way of healing achieves the desired result more rapidly is a question that has not been settled for the time being.

Do the findings in the dog equally apply to man? This question is one that preoccupies our group very much at present. However, despite occasional biopsy examinations, no definitive answer has yet been found. A short time ago we had the opportunity of studying the tibia of a 17-year-old girl who died 14 weeks after the surgical repair of the fracture*. The X-ray

* We are indebted to Dr. G. Pidermann for his enterprise and to Prof. E. Uehlinger, Director of the Institute of Pathological Anatomy, University of Zurich, who put the material at our disposal.

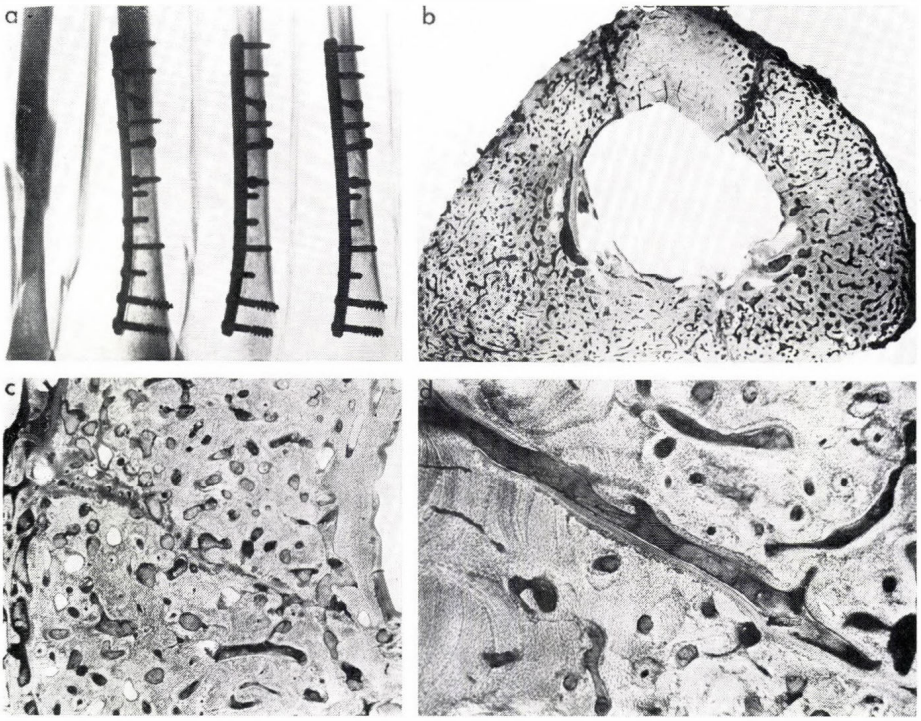


FIG. 6. Radiological and histological findings in a surgically treated fracture of the tibia of a 17-year-old girl who died 4 months after the accident. (a) Radiological findings after the accident, following surgical fixation, 6 and 12 weeks later; (b) Transverse section through the tibia. General view (cf. text); (c) Fracture gap consolidated by bony tissue. Towards the medullary space adaptation was perfect; a slight gap remained subperiosteally; (d) Section removed from a fracture gap filled by lamellar bone and demonstrating the development of osteons, which run perpendicular to the longitudinal axis. These are replaced later by longitudinal osteons but prove that the ossification in the open gap has taken place under completely stable conditions

photographs and some interesting histological findings are shown in Fig. 6. A general view of a transverse section through the tibia (Fig. 6a) reveals the conspicuously slight extent of periosteal and endosteal reaction. In contrast, intensive Haversian reconstruction is going on within the compact bone. This particular section provides a good basis for the assessment of the processes involved, inasmuch as the point of a wedge-shaped fragment can be seen at the anterior edge of the tibia. This fragment is still largely devitalized and its structure shows the situation as it was prior to the fracture. The narrow canals in the inactive Haversian systems contrast strikingly with the enlarged, darkly stained lumina in the osteons in the remaining area of compact bone, which is in the process of reconstruction. The fracture gaps have already been filled by osseous material and the regenerating Haversian canals are already moving gradually through this

new tissue into the devitalized fragment. Hence there can be no doubt that the fracture considerably intensifies the process of Haversian regeneration in compact bone, in man as well as in the dog.

The histological examination of this case gave us an opportunity also to find out what degree of anatomical accuracy can be achieved in the reduction of a fracture. The saw-cuts produced by osteotomy are, of course, not comparable with a natural fracture. Upon microscopic examination we were, therefore, surprised to discover fundamentally the same pattern as we had already observed in osteotomies performed in the dog. In circumscribed areas an effective surface-to-surface reduction had been produced (Fig. 6c), although at most points there were slight gaps, usually less than 1 mm wide. Gaps of this order, however, are filled directly by bone under the stable conditions created by internal fixation. In some places it could be shown that primary lamellar bone is deposited on the fracture surfaces (Fig. 6d). This leads to the development of the characteristic transverse osteons not infrequently seen in our experiments with the dog. There is, thus, no reason to doubt that the histological criteria of primary healing inferred from studies in the dog are equally valid in human fractures. The findings described fully confirm the hypothesis that the differentiation of sustentacular tissue is very largely influenced by mechanical factors.

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Symp. Biol. Hung., 7, pp. 87—98 (1967)

BRIDGING OVER OF LARGE DIAPHYSEAL DEFECTS

(CLINICAL AND EXPERIMENTAL RESULTS)

by

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In cases of malignant tumours the surgeon often has no other choice than to perform amputation of the involved limb. When there is a benign but very extensive bone tumour, the decision of the surgeon may be even more difficult. The destruction of the supporting bone may be so great that radical operation can be achieved only by resection of the diaphysis.

Among the most frequently occurring benign tumours are the osteochondromas which destroy the shafts of long bones both in circumference and in length. By a radical resection up to 20 cm long defects can arise. Bridging over of such a large defect is illustrated by one of our cases operated in 1953.

The patient (a man aged 40) had an enormous osteochondroma in his humerus. The circular destruction of the diaphysis is demonstrated on the roentgenogram (Fig. 1). The only chance of cure was offered by resection and subsequent filling up of the 16 cm long defect by an autoplastic fibula transplantation according to the principle of *fibula pro humero*. Postoperative treatment was carried out for four years on account of repeated fractures of the graft (the first fracture occurred in sleep, the second one when the patient leant on his elbow). Though the end result showed healing of the fibula (Fig. 2), its resistance to mechanical loading is still problematic. Owing to partial resorption, the fibula became thinner than originally.

Similar extensive resorptive processes occurring in large cortical grafts have often caused failure of our interventions.

In another case (a woman aged 20) resection was performed on account of osteoclastoma of the ulna (Fig. 3) and subsequent bridging over the 8 cm long defect with cortical bone graft. During three months of immobilization in plaster, extensive resorption of the implanted bone graft occurred leading to poor end result (Fig. 4).

In bone tumours termed 'semi-malignant' by Zollinger (1946), often extensive resection must be performed since final healing can be achieved only by such radical surgical intervention. This applies to giant-cell tumours, multinuclear fibromas and osteochondromas.

Primary malignant bone tumours, fibrosarcomas, chondrosarcomas, Ewing's sarcomas, etc. can also be healed by resection, provided they are recognized in their earliest phase. Bauer (1960) reported a case of fibrosarcoma of the tibia operated by himself. After an extensive resection, the defect was bridged over by fibular autotransplantation. The treatment

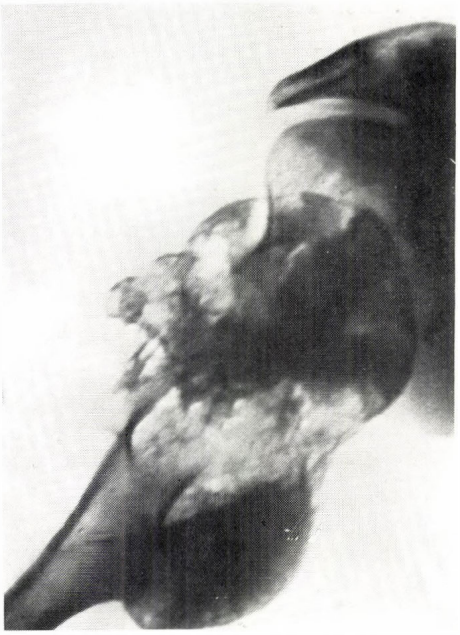

FIG. 1. Osteochondroma of the humerus

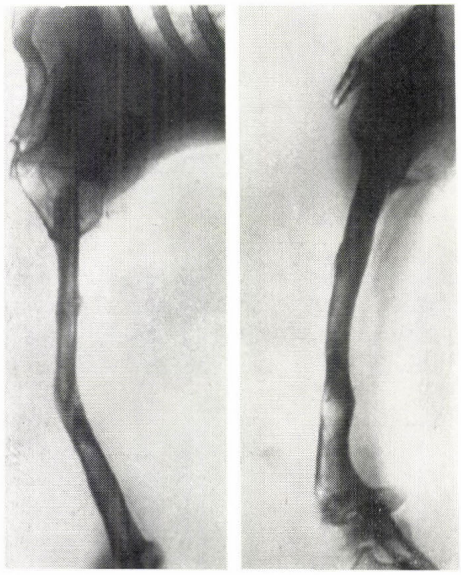

FIG. 2. Same case. Autoplastic fibula transplantation. Good healing but poor resistance to loading

lasted for four years and repeated bone graftings were needed because the fibula alone was unable ot support loading. According to Hellner (1958, 1960, Hellner et al. 1963), resection is possible in the femur or tibia in cases of osteogenic sarcoma, but the filling up of the defect arisen following excision of the tumour is technically impracticable. Therefore, the only chance of cure is afforded by early amputation with preoperative and postoperative radiation.

Undoubtedly, several sarcomas of the long bones have been definitely cured by early resection. We also have a follow-up case since 15 years, when a 14 cm long tibial resection had to be performed on account of osteochondro-sarcoma. Fourteen years ago the defect was bridged over by a strong cortical graft but with bad result. The bone graft was gradually resorbed leading to the development of a fistula and finally it had to be removed as a sequestrated bone.

The necessity of resection cannot be questioned in benign and semimalignant bone processes, since by this operation the tumour disease can be cured. The reconstruction and bridging over of the defect created by resection remains, however, an open technical problem which is still to be solved.

More than ten years ago we have attempted in the Clinic of Surgery in Zagreb to bridge over shorter diaphyseal defects using preserved cylindrical compact cortical bone. The homogenous grafts were kept in the bone bank at — 25 °C. In all cases operated in this way, a very slow postoperative bone growth was observed associated, however, with extensive

osteolysis. The end results of these interventions were mostly unsatisfactory. After various considerations we concluded that new technical modalities must be developed in cases of massive bone transplantations.

In 1960 a young man aged 20 was operated on because of a benign bone tumor in his right humerus (Fig. 5). According to radiographic evidence it was an osteochondroma which was later verified also histologically. Resection was performed and the 12 cm long defect was bridged over by implanting in the cavity a cylindriform tibial graft of the same length (cold preserved). Since during the long period of bone reconstruction partial resorption was to be expected, we did not employ humerus for grafting but tibia diaphysis because it is thicker than the humerus. To facilitate bone growth to the graft about 25 holes were bored in the cylinder-shaped bone with a drill 4 mm dia. Fixation was performed with a Rush pin (Fig. 6), and a postoperative abduction splint was applied for four months. Two years after operation the bone was firmly consolidated. The movement of the shoulder

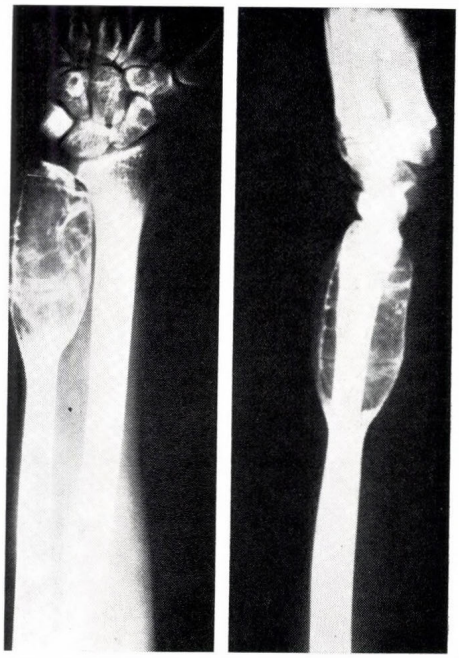

FIG. 3. Osteoclastoma of the ulna

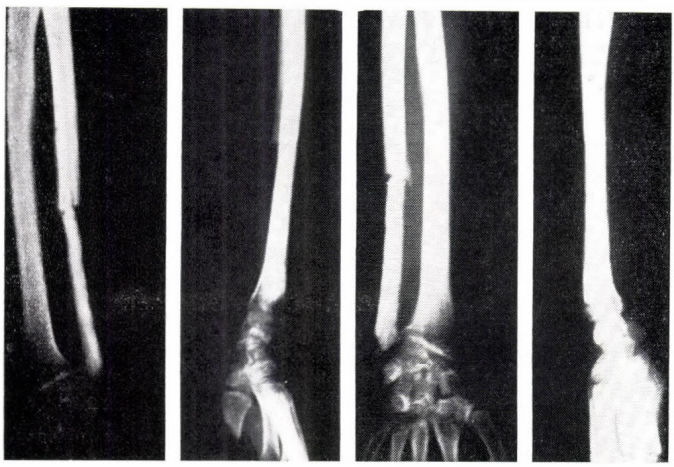

FIG. 4. Same case. Left: bridging over of the defect created at resection by a cortical bone graft. Right: 3 months later, resorption of the implanted graft. Poor end result

FIG. 5. Osteochondroma of the humerus

and elbow joints were normal. The patient, without our knowledge, recommenced his training as an amateur boxer. Four years after operation the excellent function was unchanged. The radiograph (Fig. 7) shows that the bone graft has fused with the bone. It is not so voluminous in size as four years ago owing to partial resorption, and the thickness of the tibial cylinder now corresponds to that of a humerus. Most of the drilled holes are filled with cortical bone. A few holes persist unchanged. They presumably serve for the newly formed vessels to penetrate into the inside of the bone. Percutaneous angiography of the right brachial artery has also been performed. The radiographs were taken with internal and external rotation of the arm and with and without compression of the cubital artery (Figs 8 and 9). The radiographs show hypertrophy of the aa. circumflexae humeri and a specially dense periosteal vascular network around the graft. This hypervascularized area extends to about half of the proximal part of the transplant. The superficial branches of the medial and radial parts are somewhat more voluminous, especially at the distal end of the

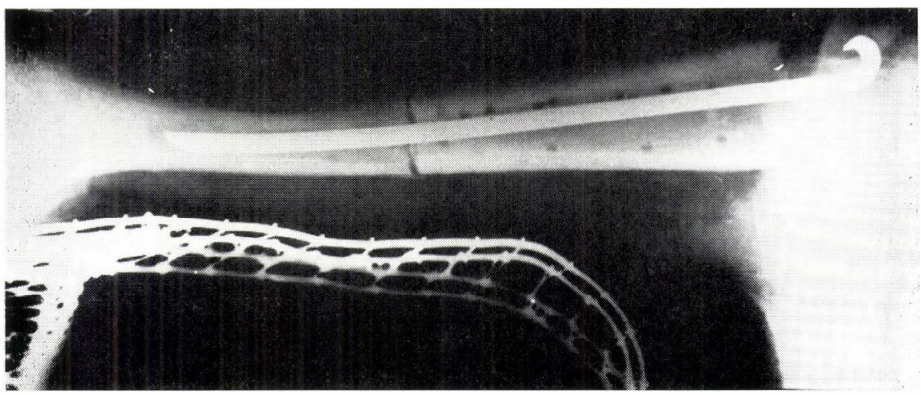

FIG. 6. Same case. Bridging over of the defect arisen after resection by a perforated tibial graft. Fixation with Rush pin

graft.*The theoretical basis of such rapid fusion of very long, perforated bone transplants has been described in numerous publications of Willenegger (1964), Basset and Creighton (1962), Allgöwer et al. (1963), Axhausen (1962) and others. By preservation in cold, the cortical splinter transforms into a homeostatic transplant which scarcely has any immune-biological activity or grows quite indifferently into the host bone (Willenegger 1964). We employ grafts only after six months of preservation in cold. The implanted dead cortical bone becomes slowly grown through by osseous elements. It is remarkable how the invading mesenchyma makes use of the openings of the bone (Willenegger 1964). Cancellous bone with its innumerable pores has long been found to be specially adapted for such purposes in clinical practice. The cortical bone on the other hand, is almost devoid of openings or canals, so that the invading mesenchyme must first form an opening for itself. The cortical barriers are being attacked by osteoclasts bringing about corrosions on the margins which are initially indiscernible on the X-ray picture. By histological examination, these lacunae contain new osteons and osteocytes building primary lamellar bone.

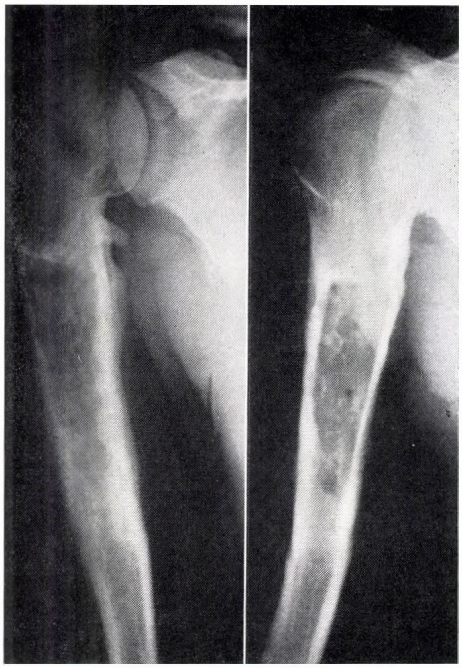

FIG. 7. Same case. The bone graft has fused with the bone. The drilled holes are filled with cortical bone

The cortical implant is slowly grown through because the young mesenchyma of the bed is not sufficiently strong to break through the cortical barriers. Under unfavourable circumstances osteogenesis can be entirely repressed by osteoclastic bone resorption. This is the cause of the poor results frequently seen following such operative interventions. Therefore, optimal conditions must be ensured, i.e. perforating the cortical implant, described above, by which the way for the mesenchyma will be open. Nature alone cannot do this without our help. We must ensure the same favourable conditions with cortical bone implants as are given with cancellous bone without depressing its mechanical function. The homeostatic principle of the osteoconductor should by all means be respected.

On the basis of these considerations, a better solution of the therapeutical problem of osteoclastomas of the femoral and tibial condyles

* I wish to express my sincere thanks to Prof. G. Gvozdanovič and Dr. M. Vidovič for the radiographs and performance of angiography.

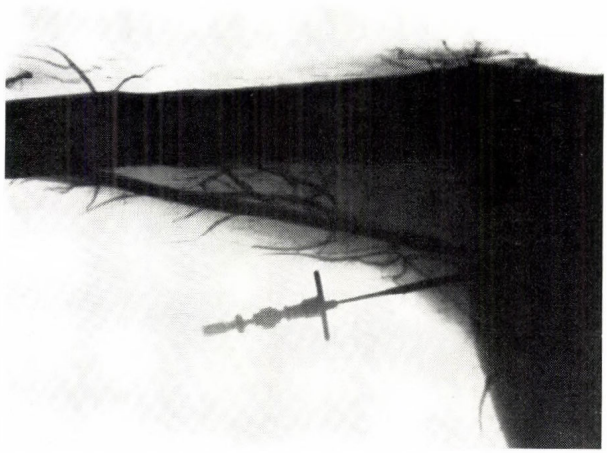

FIG. 8. Same case. Percutaneous angiography of the right brachial artery. Internal rotation of the arm. Compression of the cubital artery

seems to be available. The methods of knee-joint resection, according to Hellner (1958, 1960, Hellner et al. 1963), used so far, are not satisfactory either technically or concerning the end results. In such prolonged processes it would be preferable to resect the whole knee joint (Fig. 10). A defect up to 15 cm in length can be bridged over by a perforated cylindriform bone graft. After adequate perforation, medullary nailing is performed using a 60 to 70 cm long Küntscher nail 14 to 16 mm dia.

Basset and Creighton (1962) experimentally verified in dogs that cold stored cortical bone of calf was more rapidly grown through by osseous elements than an autogenous cortical bone graft. Basset attributed this to the much broader capillary network present in the calf bone than in the dog. Therefore, he suggests that lyophilized calf bone would give more satisfactory results in man than does a homogenous graft.

Recently, Küppermann and Schwier (1964) advocated the use of perforated preserved cortical bone grafts. In their opinion if perforated grafts are used, the surface of contact with the bed is broader and so the growth through the holes is facilitated without diminishing the stability of the graft.

In comminuted fractures with larger dislocated bone fragments devoid of periosteum, such a fragment can be regarded as an autotransplant, especially

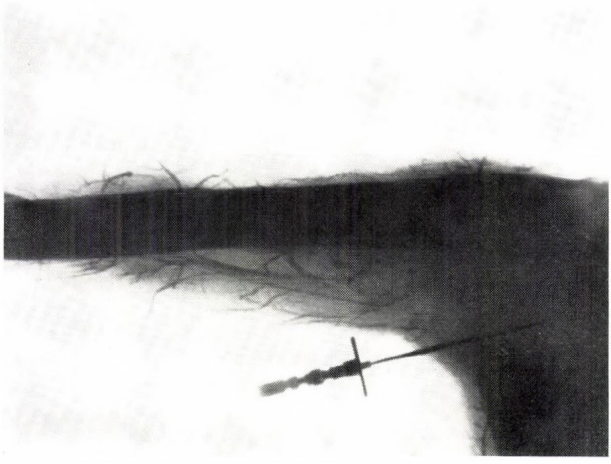

FIG. 9. Same case. Percutaneous angiography of the right brachial artery. External rotation of the arm; no compression of the cubital artery

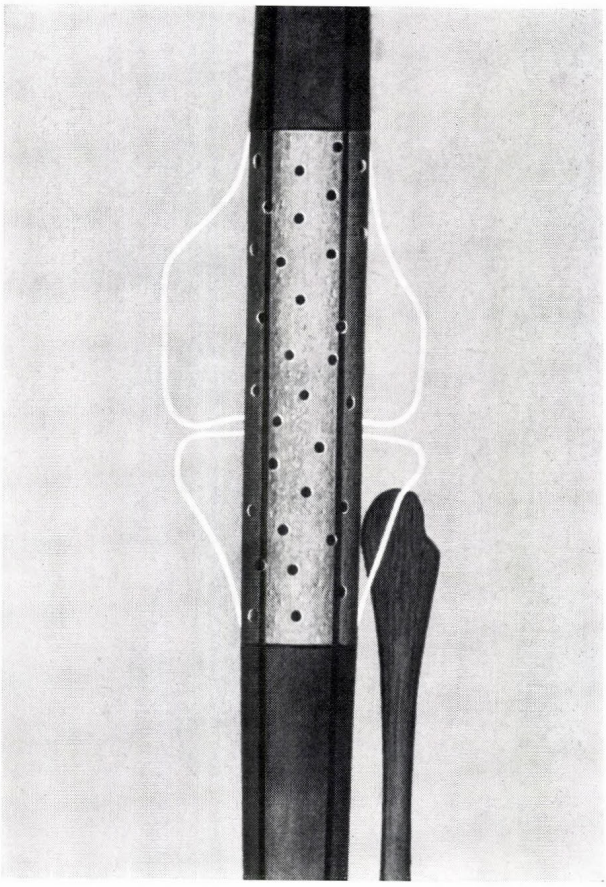

FIG. 10. Resection of the whole knee-joint and bridging over the defect by a perforated cylindriform bone graft. Fixation by medullary nailing

when osteosynthesis is being carried out. The reduction of such a bone fragment is nothing else than an autogenous transplantation. Here, too, the conditions of the expected organization must be ensured. Each piece of cortical bone being longer than 2 cm must first be perforated. In such cases as suggested by Müller et al. (1963) the cortical fragments must be removed and replaced primarily by spongious bone grafts.

Our encouraging clinical experience with perforated transplants and the rather unclarified experimental data prompted us to perform experiments in order to elucidate certain questions. We wished to investigate the following points:

1. The extent to which the mesenchyma of the graft bed resorbs the implanted cylinder-shaped homogenous graft;
2. The existing conditions of osteogenesis here;
3. Whether the relation osteogenesis—osteolysis changes with perforated grafts;
4. Whether the perforated bone graft is advantageous.

The experiments were carried out on dogs using the following methods.

In a series of dogs a 6 cm long resection was produced in the diaphysis of the radius.

The diaphyseal defect was filled up with a preserved homogenous graft of identical size.

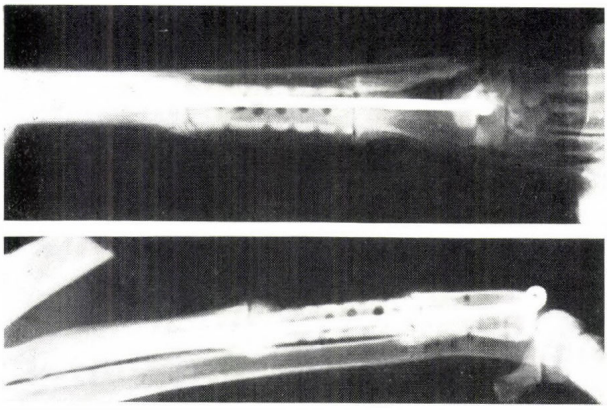

FIG. 11. Transplantation with perforated compact bone graft in dog; 79 days after operation. No osteolysis is visible; the graft is firmly fixed to the living bone

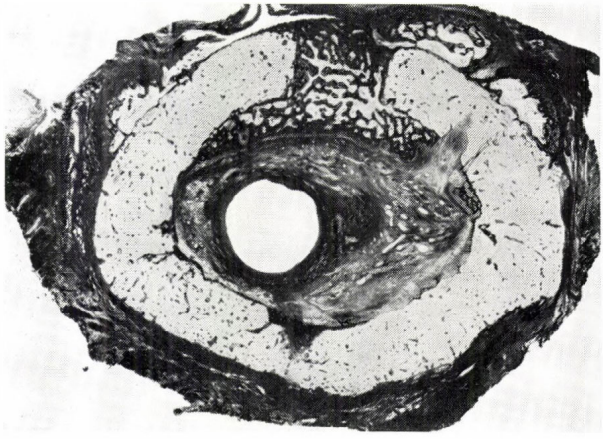

FIG. 12. Same case. Cross-section from the proximal third of the graft. New osteons have grown in the compact bone. Revitalization initiates through the holes spreading to the inner side of the graft

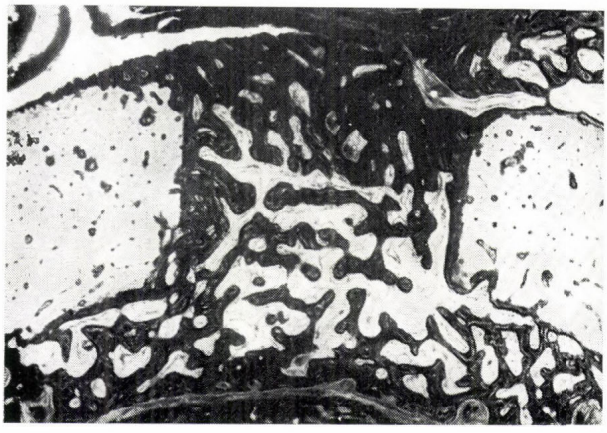

FIG. 13. Portion of the same section shown in Fig. 12 with greater magnification

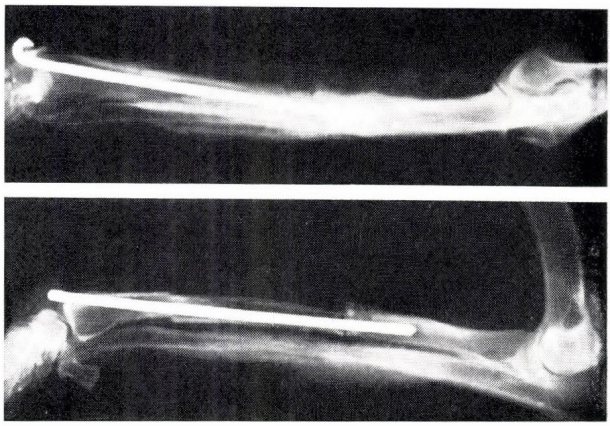

FIG. 14. Transplantation with unperforated compact bone graft in dog; 79 days after operation intensive osteolysis, resorption and destruction of the graft

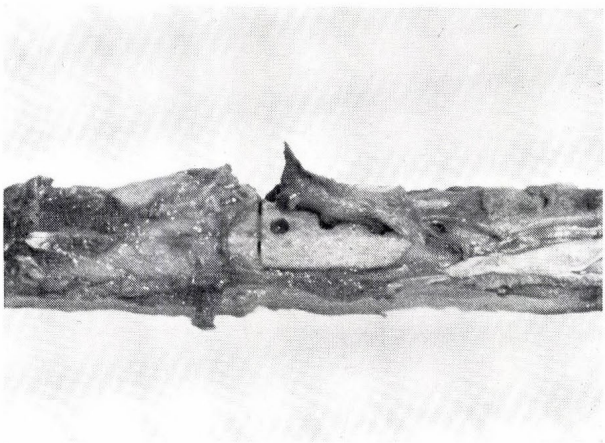

FIG. 15. Gross anatomical specimen of a perforated graft; 34 days after transplantation firmly fixed mesenchymal warts in each hole

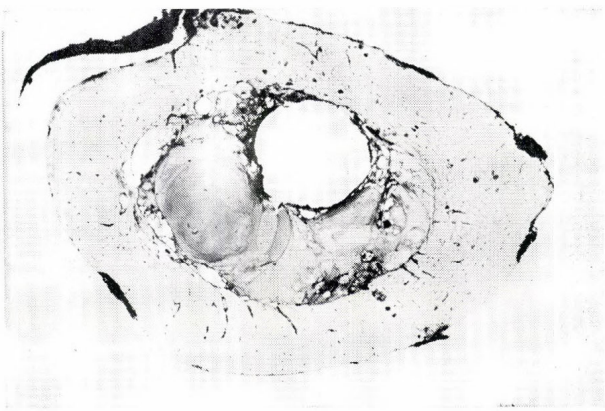

FIG. 16. Same case as in Fig. 15. Histological preparation showing some peripheral lacunae filled up with bony regenerate

Preservation at −25 °C of the bone used for grafting must last at least six months.

Fixation of the graft was made with a Rush pin.

The leg was splinted for four weeks. In one group of dogs compact bone grafts were used, while in another the transplants were perforated.

The dogs were sacrificed in pairs. The dissected material was examined by gross anatomical inspection, radiography and microscopical examination of histological preparations. For technical reasons (disease of dogs) the analysis of only 5 dogs was possible.

According to the results obtained from these experiment the following conclusions can be drawn:

(a) the perforated transplants are affected by resorptive and osteolytic processes to a much smaller extent than the unperforated ones;
(b) the surface of contact of the transplant with the invading mesenchyma is considerably increased by the holes drilled in the bone. The mesenchymal warts penetrate as roots into the bone;
(c) with compact grafts osteolysis is accelerated and more intensive than osteogenesis. The stability of the transplants is considerably diminished.

We wish to present some figures to illustrate the results obtained in our experiments.

Dog No. 1. Transplantation with perforated compact bone graft. Wound healed by first intention. The dog was sacrificed on the 79th day after operation, and X-rayed on the same day (Fig. 11). No osteolysis is visible in the transplant. Proximally and distally the graft is firmly fixed to the living bone by an abundant callus. In the middle portion of the graft no signs of bone formation can be observed. The perforations—compared with the first X-ray pictures—have become cornet-shaped. Figures 12 and 13 show cross-sections from the proximal third displaying a callus filling the hole. New osteons have grown in the compact bone. Revitalization initiating through the holes spreads to the inner side of the compact bone.

Dog No. 2. Operated on the same day as dog No. 1. Transplantation with unperforated compact bone graft. Roentgenogram taken on the 39th day discloses signs of osteolysis and resorption. The dog was sacrificed on the 79th day after operation and X-rayed on the same day (Fig. 14). The roentgenogram shows intensive osteolysis, resorption and destruction of the graft with concomitant irregular corrosions and vigorous periosteal bone building in the ulna.

Dog No. 3. Operated on both forelegs implanting an unperforated bone graft in the left leg and a perforated one in the right. The wound healed by first intention. Sacrificing occurred on the 34th day after operation. The roentgenogram taken on the same day discloses bilateral formation of small periosteal spurs at the surface of contact. Postmortem examination of the material revealed that a mesenchymal tube was present around both transplants. A longitudinal incision on the unperforated transplant shows a smooth surface of contact with the mesenchyma, whereas the incision of the mesenchymal mantle of the perforated graft (Fig. 15) disclosed firmly

fixed, ingrown mesenchymal warts in each hole. The histological preparation (Fig. 16) shows the cross-section of some peripheral resorption lacunae filled up partially with bony regenerates.

SUMMARY

Benign and semi-malignant tumours of the bone often destroy the diaphyses in their total circumference. Radical elimination of the tumour can be achieved only by resection of the diaphysis extending proximally and distally to the intact bone. The reconstruction and establishment of continuity of the diaphysis required after such resections is still an open surgical–technical problem. Bridging over the defects with cortical bone grafts failed to bring satisfactory results, owing chiefly to resorptive and osteolytic processes occurring in the graft which lead to premature destruction of the graft. According to personal experience of the authors, the consolidation of the graft can be considerably accelerated and improved if the cylinder-shaped cortical transplant has been previously abundantly perforated. Through these perforations the mesenchymal tissue of the graft bed is able to invade directly the homeostatic graft. In this way, the preparatory osteolytic processes of the cortical barriers are not needed. Experiments carried out on dogs have confirmed the advantage of perforated grafts.

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Symp. Biol. Hung., 7, pp. 99—104 (1967)

CONTRIBUTION TO THE LATE RESULTS OF GRAFT IMPLANTATION IN THE BACKBONE

by

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THE SURGICAL removal of one specific tuberculous focus of the backbone is a routine oparation nowadays. The 'where' and 'when' of this operation as regards the removal of the centre of the infection or of the specific abscess are, in general, connected with compression symptoms, paravertebral and psoas-abscesses or bone sequesters. Obviously, the removal of the focus, principally in cases of great destruction, raises the question of vertebral stiffening or of the replacement of the backbone. These methods are, however, acceptable only when the defect of the backbone can be replaced for a long time, i.e. when surgical removal can help in restoring health.

For many years we have been engaged on this problem. Our previous experience was published in 1954 (Schnitzler and Fábián). The essence of our method is to fill up the gap of the backbone with bone, the defective part of the vertebra being thus substituted by a bone transplant. In all cases the graft is resected (including the periosteum) from the crista iliaca of the patient. The cavity of the bone is filled up with Orell's bone chips and the defect is filled up with a bone bridge, the size of a little finger, for better stabilization of the backbone. In cases when resection of a large portion of the vertebra is necessary, the bone block obtained from the crista iliaca should be formed in such a manner as to fill up the whole defect. The bone should be placed in the defect in a strong lordosis of the spinal column, in order to have it under compression in a physiological position. Frequently, several adjoining vertebrae have to be removed; as a result a large defect arises the size of 2 to 3 vertebrae. In such cases it is necessary to form column-like grafts and place them lower and higher than the defect into the intact part of the backbone.

Since 1954 we have performed the operation mentioned above, on 46 patients. Follow-up examinations have been carried out on 9 patients for more than 10 years, and the period of observation regarding 24 other patients is approximately 6 to 9 years. Our clinical results are excellent. In our opinion our good results are due to our surgical technique and method of transplantation which are based on the experimental results of Krompecher substantiating the following principles:

1. Active movement helps bone formation;
2. Functional employment promotes the survival of the transplant (Krompecher and Pap 1963);

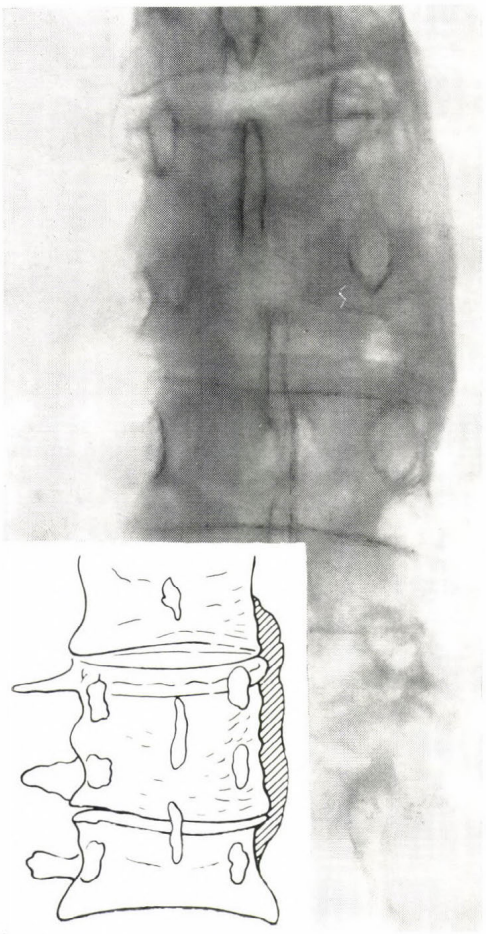

FIG. 1

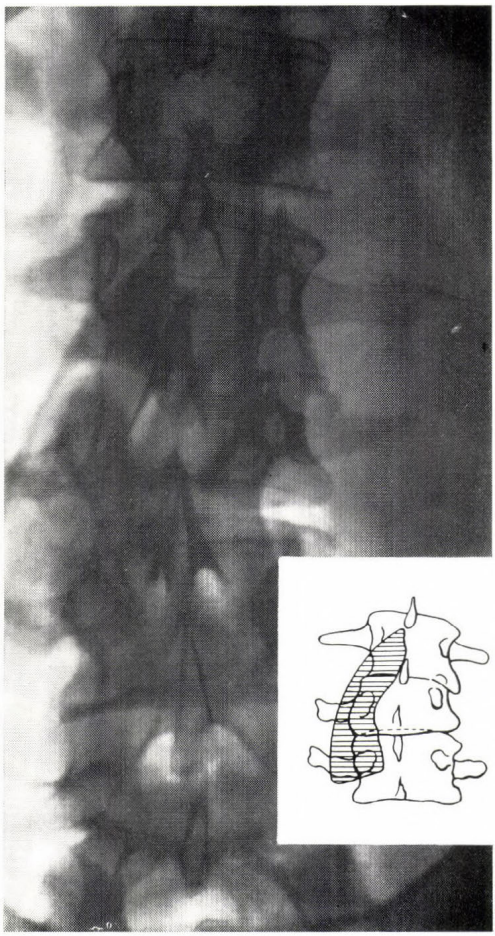

FIG. 2

3. Inactivity stimulates the resorption of the graft (Krompecher and Pap 1963);
4. Unnecessary transplants are also resorbed.

On studying such an operated case, the first finding is the fracture of the spinal column. This artificial fracture is united by the surgeon by means of a bone transplant, and the subsequent result is due to the natural force of regeneration. Opinions concerning this problem are not uniform. There are specialists who uphold the view that the formation of callus in the vertebrae is generally minimal. Lob (1954) claims that uniting the fracture of the spinal column by a callus is impossible. According to Somogyi et al. (1956), X-ray examinations reveal that the lines of a coarse fracture of the

vertebrae are persistent even after several years following the injury. Budai's opinion is that the fusion of the vertebrae occurs only by connective tissue, and a bone block can only develop if the ligament was also damaged. There is another difficulty in these cases. The graft must be placed into a tuberculotoxic environment. The question is how callus formation and regeneration occur under such unfavourable tissular conditions. According to Lob (1954), first the connective tissue is developed, and fusion of the vertebrae by the implanted bone comes later. Kastert (1957) states that after the removal of the specific focus, the bone block consists of connective tissue or of bone. In such a case there is no chronic inflammation *in situ*. The new bone develops only in the presence of a simultaneous chronic inflammation of the tissue. According to Erlacher (1935), it is probable that the unsatisfactory nutrition of the bones, due to the pathological condition of the tissues, greatly disturbs the formation of callus. Borsay et al. (1959) declare that scarcely any callus formation exists in the vertebrae.

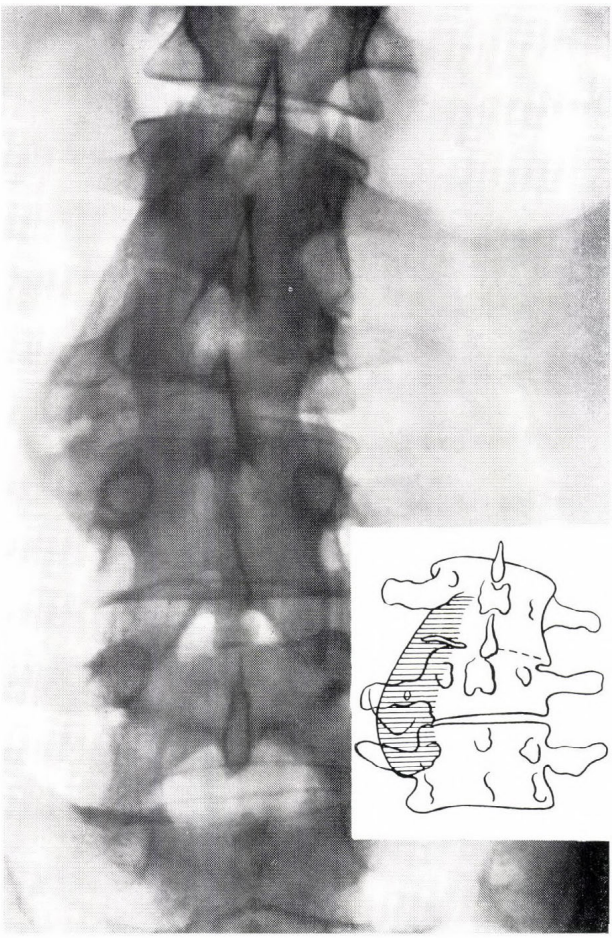

FIG. 3

These data do not offer any satisfactory explanation as to our very good results. Therefore, an analysis of our material seems to be necessary. It should be noted that our patients have been living and working practically without complaints for about 10 years since their operations, which fact seems to verify the correctness of our surgical method.

1. This woman patient was operated 11 years ago because of spondylitis tuberculosa of the vertebrae lumbalis II and III. A relatively large defect was created by the removal of the focus. The gap was filled up with bone chips and the two diseased vertebrae were united with a bone graft the size of a little finger. The clinical state of the patient is excellent today. The gap does not exist and the bone block is bridged by a well-developed callus. On the external border of the bone block, at the site of the transplanted periosteum, a large cortical layer was formed (Fig. 1).

2. The vertebrae lumbalis II and III of a young girl were removed, and the gap was spanned with a big bone bridge. A portion of the transplant reached with the periosteum down into the soft tissular environment (Fig. 2).

Today, 11 years after the operation, a well-developed bone block exists. The unobstructed part of the bone transplant, mentioned above, is, however, broken and partially resorbed (Fig. 3).

3. Removal of the focus was performed between the vertebrae lumbalis II and III. The gap was filled up with a well-formed bone block with periosteum the size of 1 × 6 cm (Fig. 4). Today, after 10 years, a well developed bone block is visible, but the border of the periosteum has not disappeared as yet. The gap exists only where the discus persisted.

4. In this case the vertebrae lumbalis III and IV were removed. The removal of the lateral parts of the two vertebrae extended far into the deep interior of the backbone. A triangular graft was implanted with periosteum into the gap. After 10 years, a big callus is still visible in the vertebra (Figs 5 and 6).

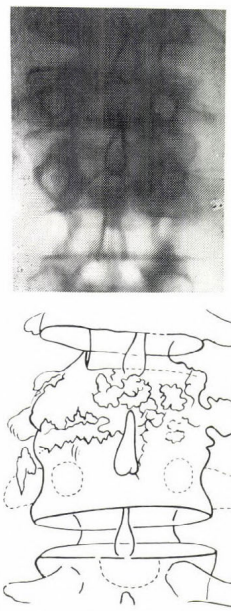

FIG. 4

5. The operation on this woman was performed 11 years ago. The vertebrae lumbalis III, IV and V and sacralis I were removed because of spondylitis. A larger part than half of the two vertebrae was sequestered. The backbone was united at the external border with a big bone column with periosteum. On this transplant a well formed functionally valuable callus has developed. On the border of the periosteum a cortical layer still exists (Fig. 7).

We have performed the operations, mentioned above, on about half of our patients with spondylitis dorsalis. Our results in this group are practically the same; the period of observation is, however, less than 10 years. In cases of spondylitis dorsalis, the X-ray pictures are not so convincing as in spondylitis lumbalis. This fact is undoubtedly a further difficulty in the assessment of the results. Moreover, in cases of spondylitis dorsalis much larger grafts are needed for implantation.

According to our results, we are of the opinion that autotransplantation is a very good method for

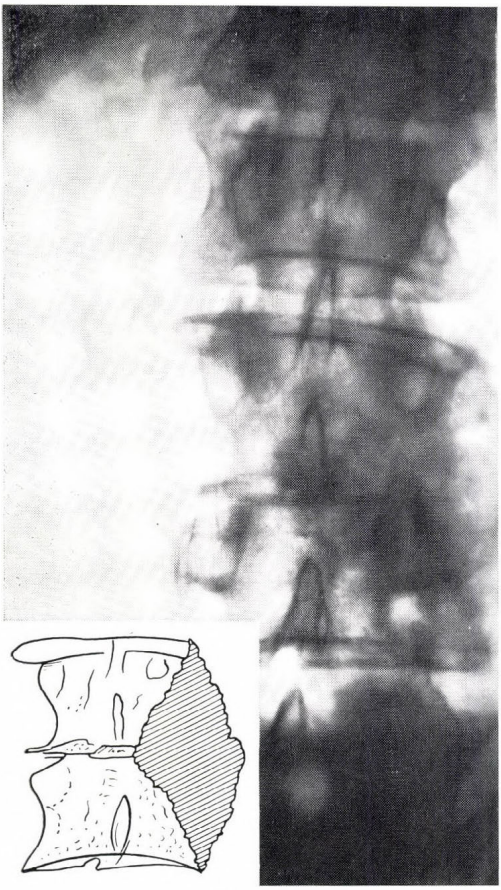

FIG. 5

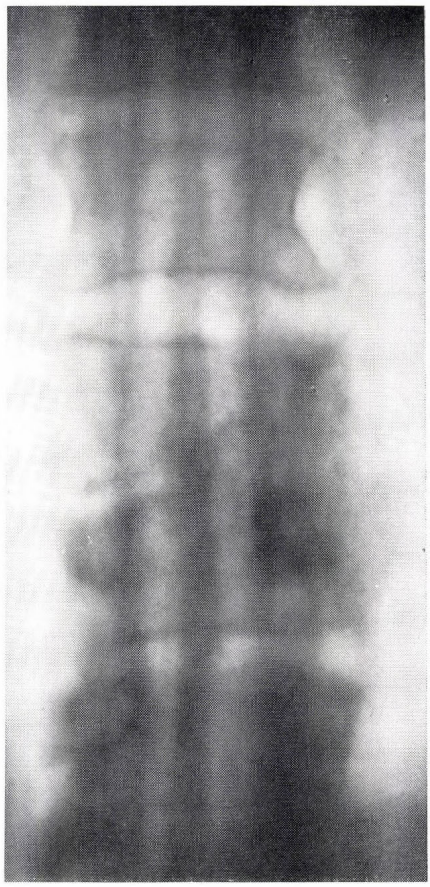

FIG 6.

the medical treatment of spondylitis tuberculosa. The clinical condition of the operated patients, even after several years, is excellent and in every case the development of a big and well-formed callus has been evidenced. We have, however, no pathohistological documents regarding this material, because up to the present all our operated patients are alive.

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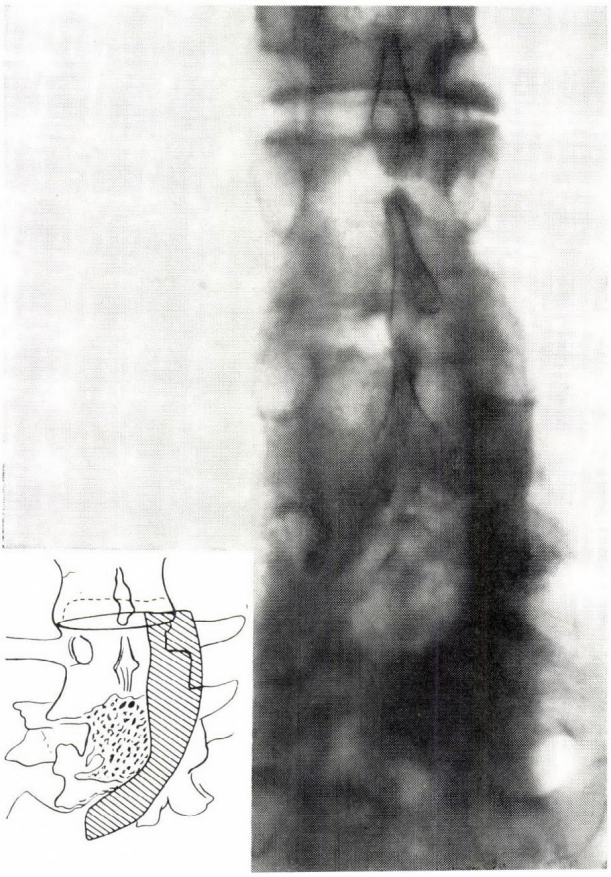

FIG. 7

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Symp. Biol. Hung., 7, pp. 105—107 (1967)

STUDY ON VERTEBRAL CORPODESIS IN DOGS

by

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According to our clinical and roentgenological observations we wrote in 1959: 'In cases of particularly severe vertebral tuberculous destructions it may be frequently noted that the stability of the vertebral column is secured only by the posterior "tube system" with the help of the small joints, the spontaneous ossification of which represents the highest degree of natural adaptation. . . . 'On analysing the bone-graft implantation into the vertebral focus we wrote the following: 'The spontaneous callus-formation or that due to operative influence is limited to the preservation of the "tube-system" and may be observed only at the circumference of the vertebral body . . . thus, the expedient surgical method or technique is that which makes use of the repairing power of the vertebral border.'

Our clinical and roentgenological observations were partly supported by animal experiments. The collaborators were Dr. Joós, Dr. Lelik, Dr. Kerényi and Dr. Varga.

In 15 dogs we tried to analyse the behaviour of autogenous bone grafts implanted on the side of the vertebral bodies. In one case we operated the dorsal vertebrae through costotransversectomy. However, the surgical procedure was not adequate technically for serial experiments in dogs. On one occasion we performed lumbal corpodesis transperitoneally. The animal perished due to vena cava thrombosis. In 13 cases the approach to the lumbal vertebrae was retroperitoneal and, after chiselling superficial or deeper grooves, a bone graft of the resected eleventh rib was implanted into the vertebral body. Ten animals were followed up for 2 to 6 1/2 months on roentgenograms (3 animals perished or were lost), then the affected part of the vertebral column was removed *in toto* and separate roentgenograms were taken. Histological preparations were not made as the material was lost in the histopathological laboratory.

Our observations may be summarized as follows. The callus formation begins on the side of the vertebrae adjoining the disk and becomes distinct after four weeks. On the affected part of the disk a narrowing, and on the adjoining vertebrae sclerosis may be observed. In case of superficial surgical injury and bone-graft application, callus formation is moderate, but distinct lateral and anterior links fasten the bone graft to the disk, and the other parts of the bone-graft are resorbed (Figs 1 to 5).

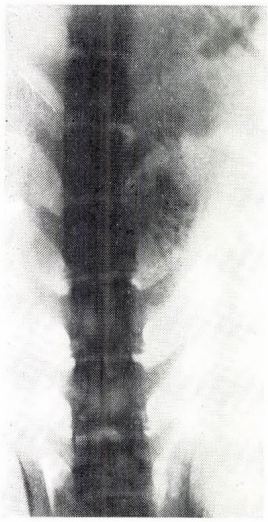

FIG. 1. In superficial operative injury, the bone graft is fixed with marked lateral and anterior bridge formation to the injured disk; the other part is absorbed

FIG. 2. See caption to Fig. 1

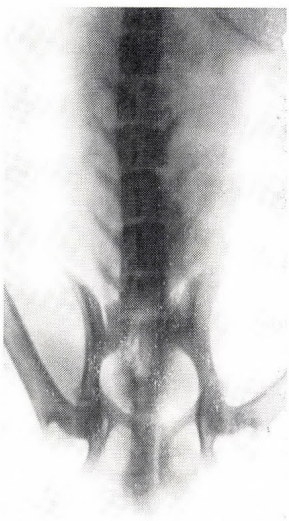

FIG. 3. See caption to Fig. 1

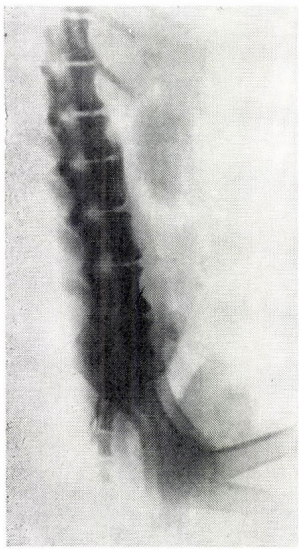

FIG. 4. See caption to Fig. 1

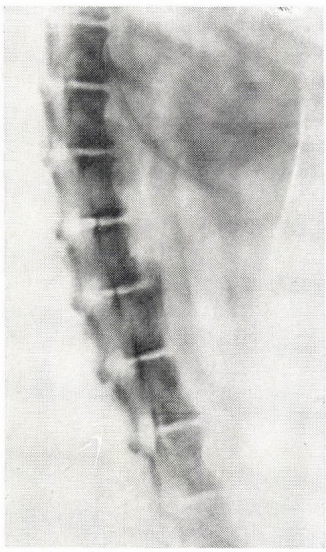

FIG. 5. See caption to Fig 1.

FIG. 6. Absorption zone around the distal end of the bone graft

FIG. 7. Extensive callus formation in case of deeper surgical injury

FIG. 8. See caption to Fig. 7

FIG. 9. See caption to Fig. 7

The bone graft which was wedged deeper into three vertebrae is consolidated after two months. The roentgenogram taken 2 1/2 months after intervention shows the same picture on the section of two vertebrae, but a small zone of resorption surrounds the end of the bone graft introduced into the third vertebra (Fig. 6).

In case of deeper and extended surgical injury the callus formation is significantly more distinct, the two adjoining vertebrae are united into a block and the bone graft is not seen on the roentgenograms (Figs 7 to 9).

According to our roentgenological examinations we may ascertain that in dogs the bone graft implanted during corpodesis is consolidated. The narrowing and the surrounding sclerosis are constant features. The callus formation begins on the side of the vertebrae adjoining the disk and after superficial surgical injury a clamp formation fastens the implanted bone graft similar to spondylitis deformans; in cases of extended and deeper injuries, powerful callus formation begins at the same site and unites the two affected vertebrae into a block. If the injury affects three vertebrae, the bony union is limited to two vertebrae only, and the bone graft beyond the two vertebrae may be resorbed.

Symp. Biol. Hung., 7, pp. 109–131 (1967)

CLINICAL ASPECTS OF CALLUS FORMATION

by

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TWO THINGS are of interest for the clinician in his everyday surgical work: whether the healing process of a fracture can be accelerated beyond physiological limits and which are the impairments retarding fracture healing. This problem can be clarified by clinical observation and by experiments on animals.

The problem of the mode of action and the role of mechanical influences and functional stimuli in fracture healing have been largely discussed. The concept, now almost universally adopted that only ideal reduction and immobilization of the fracture can lead surely to sound repair, was by no means adopted by all surgeons.

L. Chambonière in 1867 suggested the moving of fragments in fracture treatment. In contrast to this, Owen Thomas in 1887 advocated prolonged and uninterrupted immobilization based on his observation that immobilization of too short duration results in excessive callus formation. David in 1895 reported healing of replants of skull bone without callus, attributed by him to lack of muscular effect. According to David dosed rest and activity are required for healing of fractures of the extremities. Even such experienced clinicians as Lexer, do not regard immobilization as a decisive factor in fracture healing, advocating the use of adequate reduction without complete immobilization. On the other hand, in Böhler's opinion, the conditions of undisturbed healing are: manipulative reduction and immobilization of the fracture. All extensive calluses observed on the radiographs resulted from mechanical irritation of the fracture site which has disturbed the healing of fracture. According to Schenk and Willenegger, not only excessive callus formation but all calluses appearing in the vicinity of the fracture are signs of disturbed fracture healing. In experiments carried out on rabbits and dogs, we have observed that fractures in which 'ideal' immobilization was ensured achieved only a slighter breaking strength compared with the controls where less 'ideal' immobilization was performed. We all know from clinical practice cases of femoral fractures in which one year after medullary nailing, on the basis of the roentgenogram, 'ideal healing' without any callus in the proximity of the fracture would be expected and yet, after the removal of the medullary nail, refracture of the apparently well-united bone occurs at the slightest mechanical employment. The same observation was reported by Allgöwer at the Congress of

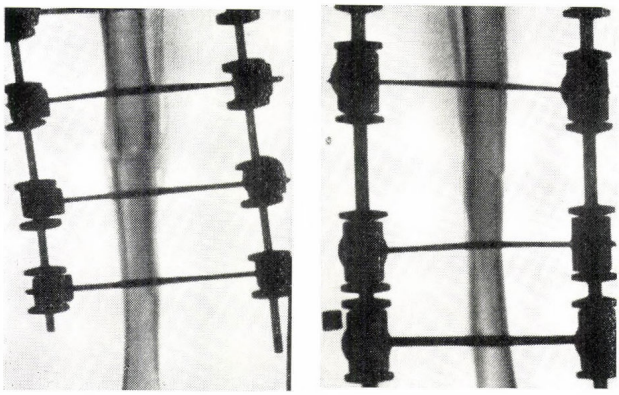

FIG. 1. Fracture of the leg of rabbit immobilized by means of an apparatus. On the right: more powerful setting device ensuring complete fixation of the fracture. On the left: small device which does not ensure adequate fixation of the fracture

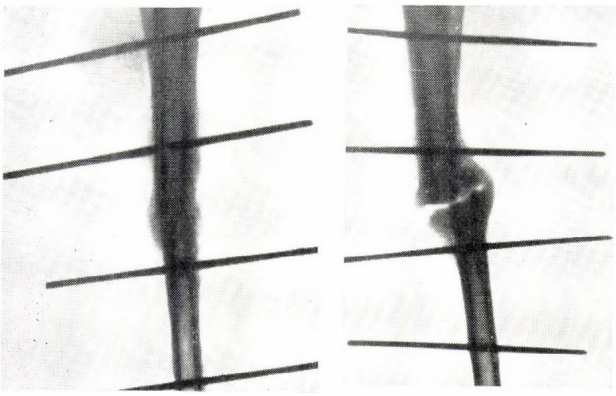

FIG. 2. Small apparatus for fracture immobilization

German Surgeons in 1964, when he distinguished between *early refracture* attributed to unsatisfactory union of the fragments and *refracture occurring after removal of metal.* According to Allgöver, the evaluation of weight bearing capacity is rendered difficult by primary bone healing. This is undoubtedly so. But this experience shows also that forced 'absolute' immobilization cannot be regarded as *conditio sine qua non* of fracture treat ment, especially if this absolute immobilization can only be achieved by great surgical-technical preparations and by simpler and less dangerous procedures similar good results can be attained. Naturally, it may be objected thatin such cases the failure of fracture healing or delayed repair may be due not to the principle of absolute immobilization but to some imperfection of the methods used to achieve this aim, e.g. too excessive tissue damage, particularly that of the periosteum, at the exposure of the fracture site for medullary nailing or for application of a compression plate. Küntscher states that whenever such a procedure is technically possible, closed stable osteosynthesis and medullary nailing without impairment of the periosteum should be performed. Continuing the fundamental experiments of Krompecher, we have found in experiments on rabbits that with ideal reduction and

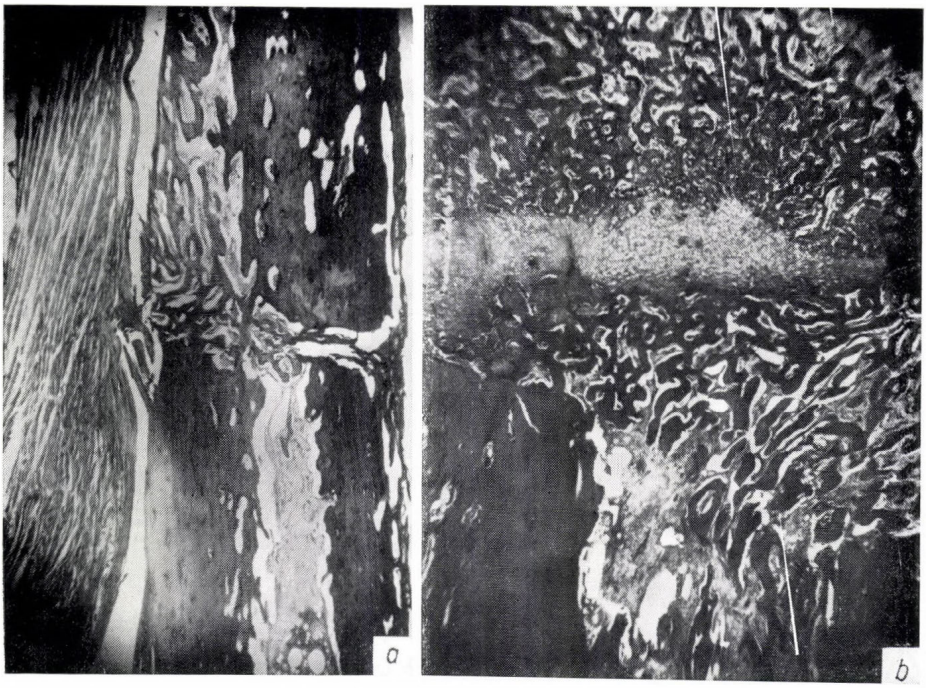

FIG. 3. During mechanical rest direct differentiation of spongious callus occurs (a). Histological section through spherical callus. Precallus consisting of cartilage and connective tissue is recognizable between the fracture ends (b)

immobilizaton of the fragment ends the healing of fracture—at least in these animals—occurs without preformation of cartilage or connective tissue and with minimal amount of callus in the vicinity of the fracture site. In such cases we speak of callus formation without indirect differentiation.

Figure 1 shows fracture of the leg of rabbit immobilized in an apparatus. On the right: more powerful apparatus ensuring complete fixation of the fracture. On the left: a small setting device which does not ensure complete protection of the fracture site against bending forces. No strong compression acted on the fragment ends in either case, they were merely brought into apposition. The radiograph taken after an observation period of four weeks, disclosed on both sides firm bony union. On the right, we see a fracture healed almost without environmental callus, whereas on the left, a larger spindle-shaped callus is visible.

In Fig. 2 two small setting devices are presented. The fragment ends on the right have not been entirely apposed and compressed to each other, while on the left, the fracture ends have been as well reduced as in the preceding picture, though without compression. On the right, a spherical callus has developed due to unsatisfactory immobilization, while on the left, a spindle-shaped callus is visible which, however, after the elapse of four weeks, will show perfect bony organization.

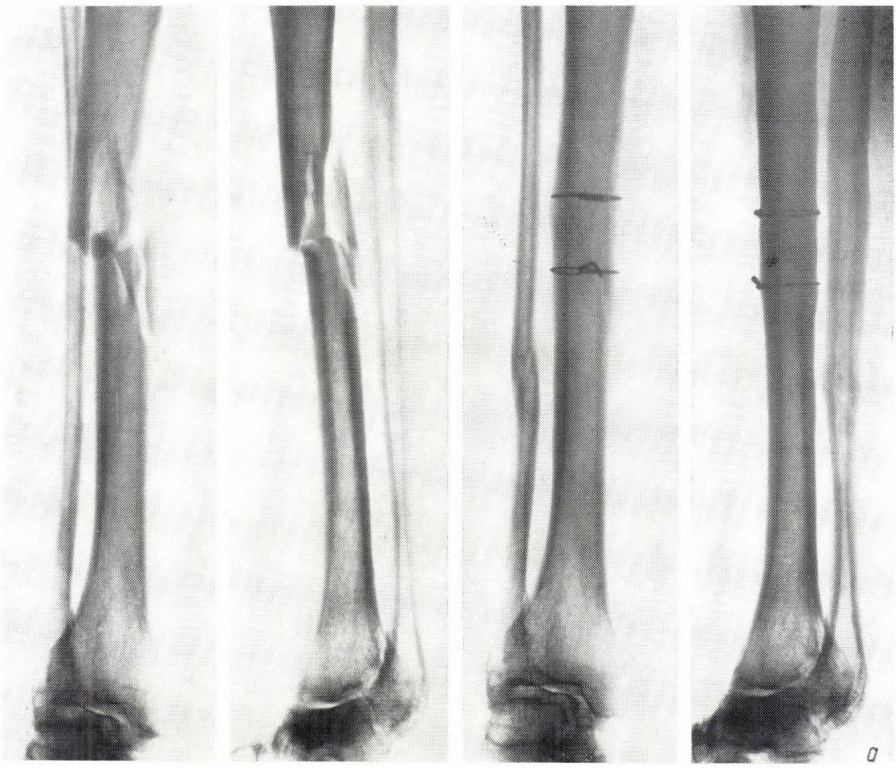

FIG. 4a. Example of fracture treated by traction and wire cerclage

From this experiment it appears that not so much 'absolute' immobilization of the fracture is required but its protection from too much movement. In this connection the experiments of Küntscher are of special interest, showing that the resistance of the blastema to mechanical employment of traction or compression is only of a moderate degree (Figs 3a and 3b).

It is difficult to achieve in experiment one-sided compression or traction exerting on the blastema. As a rule, we have to deal here with a mixture of various forces acting on it. Therefore, it is very difficult to tell which tissue has been formed under the influence of a certain force. Hasche-Klünder and Gelbke have come to the conclusion that mechanical unrest in the territory of the blastema leads in every case to formation of cartilaginous precallus. More than 100 years ago Bernhard von Heine pointed out that the periosteum, endosteum and cortical bone participate in equal measure in the healing of fractures. According to Hauck, endosteum is the main factor in callus formation, though the size of the periosteal and chiefly that of the parosteal callus is by far larger than that of the endosteal one. In Küntscher's opinion, no risk is run in renouncing formation of endosteal callus, at least in diaphyseal fractures, if the periosteum has been

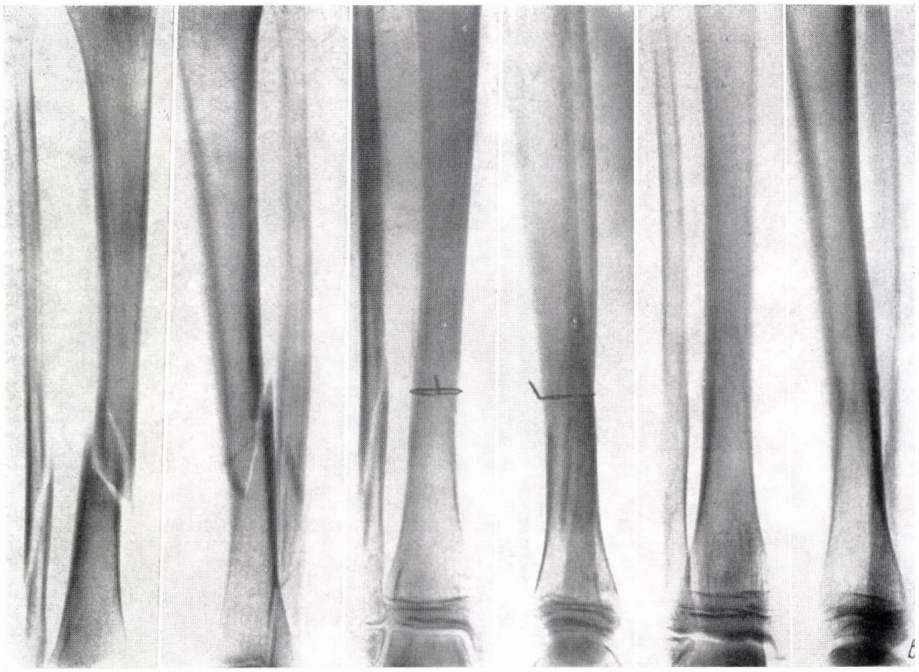

FIG. 4b. Example of fracture treated by traction and wire cerclage

carefully preserved at the operative intervention. As has been repeatedly observed in experimentally produced shaft fractures, abundant callus formation is almost consistently found in the medullary cavity near the fracture site, even if only a very thin callus spindle is visible externally on the bone. The fact that endosteal callus is not indispensable at all in fracture healing, as evidenced by clinical experiments of boring the medullary cavity and medullary nailing, does not mean that endosteal callus formation does not occur in the diaphysis.

It has been histologically demonstrated by Schenk and Willenegger that 'under stable circumstances the cortical bone can contribute considerably to the healing of fracture'. These authors pointed out that such an accurate reposition of the fragments can be achieved by compression that in some places the Haversian systems become pressed to each other. Such sites will be organized exclusively from these Haversian systems. Fusion of the fragments will occur simultaneously with re-establishment of the original structure of the cortical bone. By this, the possibility of a 'primary fracture healing' would be demonstrated. On the other hand, Geiser who likewise studied this problem very systematically believes as a result of his experiments that direct fusion of the cortical bone is impossible because of the more or less extensive necrosis always present at the fracture ends and considers the term 'primary fracture healing' as misleading. We have demonstrated

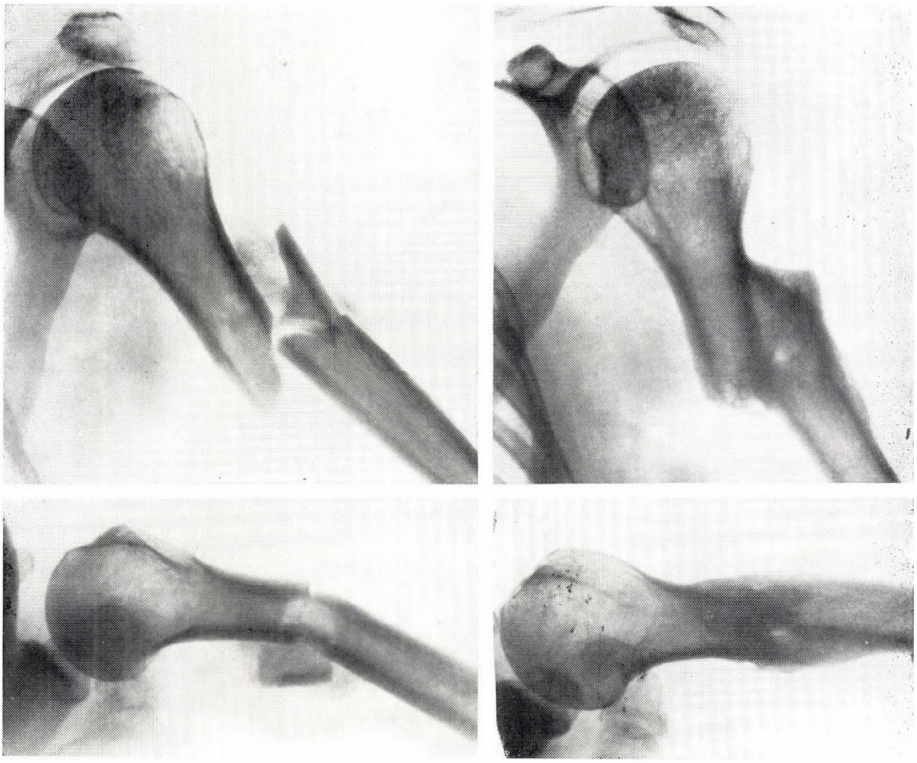

FIG. 5. Healed fracture of the shaft of the humerus with displacement as great as the breadth of the shaft

in experiments with encircling wires that the extent of necrosis occurring in the fracture ends is largely dependent on immobilization, i.e. it may be so slight in case of adequate fixation that it is practically of no importance. If immobilization has been insufficient, necrosis can assume considerable proportions which, despite wire cerclage, may lead to formation of pseudarthrosis. Allow me to break a lance here on behalf of wire cerclage. This procedure—provided it limits are known—is not so bad as its present reputation. It does not permit e.g. the disregarding of the use of plaster bandage. It would be wrong to conclude that this procedure adds the disadvantages of plaster bandage to the disadvantages of operative treatment. Much rather it promotes short and ideal adaptation of the fragments, the possibility of an early loading, e.g. that of the leg in plaster cast, and earlier dismissal of the patient from the hospital. At any rate, if wire cerclage is performed, the operation must be performed as carefully as possible. It is usually unnecessary to strip the periosteum from the bone since it has already been detached during fracture and we must limit ourselves to apply one or two encircling wires at most and never believe to have achieved a stable

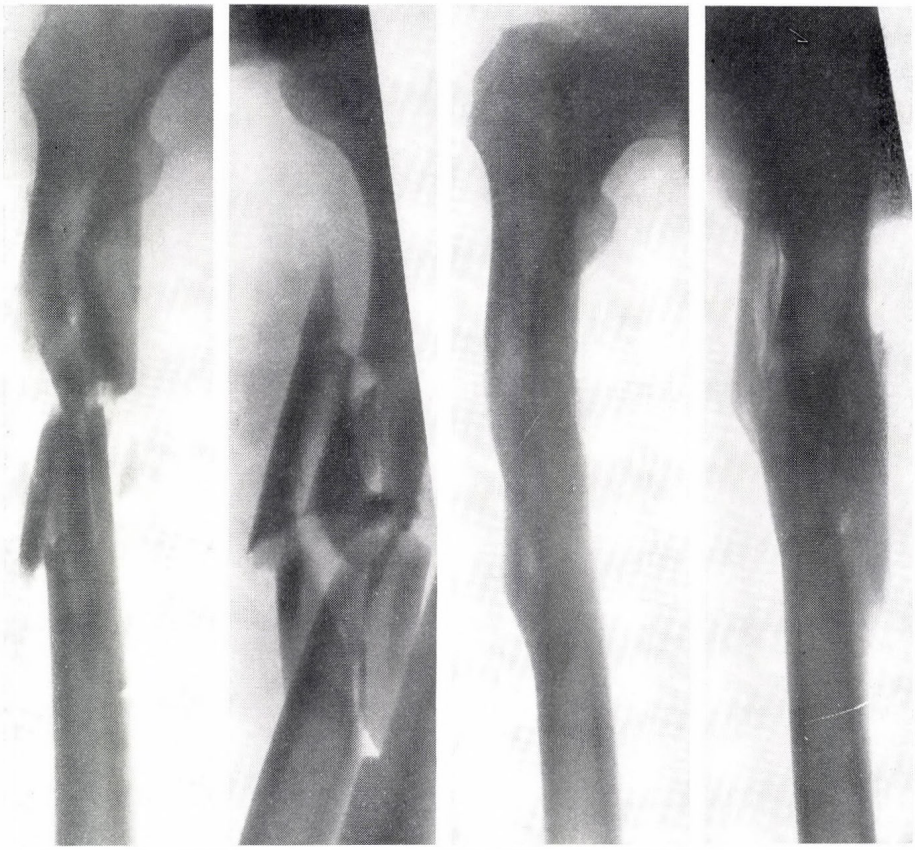

FIG. 6. Comminuted fracture of the femur

osteosynthesis with perfect weight bearing capacity. In doing so the wire cerclage would afford all the advantages of a conservative treatment without exposing the patient to the danger of introducing large metallic foreign bodies. Certainly, when one sees a radiograph where numerous loops succeed one another, one must seriously meditate over the biological considerations of the surgeon who performed the operation. Figures 4a and 4b give examples of fractures treated by traction and wire cerclage.

In recent years the mechanism of action of compression osteosynthesis, according to Westhues and Greifensteiner, though evaluated positively by most specialists, has been largely discussed.

First of all it should be stated that by pressing together the fragment ends in a fracture, osteotomy or arthrodesis, no effect is exerted on the blastema. Compression is taken up by the surfaces of contact of the bone where it dies away. In the area of the blastema itself, it is rest from mechanical viewpont, provided there are no bending or shearing forces present.

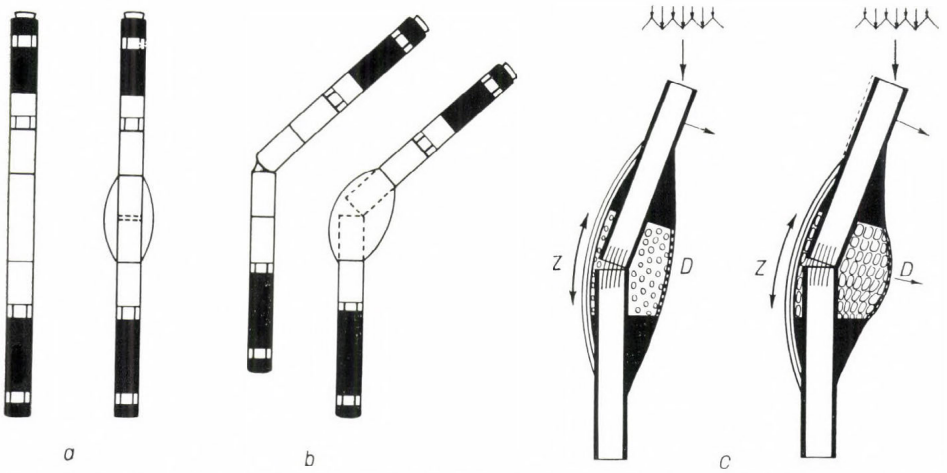

FIG. 7. Explanation of the shape of callus on the basis of hydrostatical law, according to Pauwels (a and b); Z = traction; D = compression (c)

These forces do not, however, act uniformly but produce a constant change of traction and pressure strains. If compression of the surfaces of the cortical bone had a positive effect on tissue proliferation, an increased tissue proliferation would be present at the site of contact of the cortical wound surfaces. This has not been verified so far. The very fine bone grindings of Schenk and Willenegger demonstrate direct union of bone lamellae, but nothing that would verify that the axial pressure acting on the fracture site would have brought about an excessive disposition for regeneration of the tissue. It has been stated by the adepts of the so-called 'functional fracture treatment' that the young callus tissue must be timely set to adequate functional tasks because this would promote differentiation of pluripotent histiocytes in the bone. Julius Wolff has made a sharp distinction between the process of sticking together and that of transformation, the results of which are constantly confused and misinterpreted. Wolff is right in stating that the effect of function on the process of transformation asserts itself only after completion of actual healing. Functional movement therapy is rightly considered by Böhler as consisting of complete and uninterrupted immobilization of the fracture after its careful reduction and simultaneous active exercise of the joint which is not involved in immobilization or that of the whole body, under painless conditions. When Küntscher states that full loading and active exercise of the limb is possible, setting this even as the aim of treatment, he does not mean to give a functional task to the fracture site but he trusts in the stability of osteosynthesis and in the material involved in this process. The efforts of the working team of Swiss surgeons to improve fracture healing by new procedures of osteosynthesis, especially by using compression plates, are directed not so much to obtain union of the fragments some days earlier, but to render possible the active mobilization of the joints in the vicinity of the fracture site and thus, prevent

postoperative joint stiffness. According to Müller, Allgöwer and Willenegger, the aim to be attained by the surgeon should be rather early mobility of the operated extremity than its early weight-bearing capacity. This aim has been attained by Pap, too, with his method of diafixation requiring little technical employment. In our opinion, synthesis of bone due to compression is not the work of some mysterious regenerative powers aroused there, but rapid fracture healing is due —as has been constantly observed—to accurate reduction and complete immobilization. The degree of compression differs only in so far as a more developed musculature and a longer lever arm naturally requires stronger pressure than a weak musculature and a short lever arm when one wants to achieve an adequate immobilization. Bürkle de la Camp warns mainly against using too strong pressure. Compression of about 8 kg seems to have the most beneficial effect. By applying stronger compression, bone necrosis may ensue in the surfaces of contact of the fragment ends. It has been demonstrated by Bier in his lengthening osteotomies and by Küntscher in his various communications on medullary nailing that even with distraction, provided that bending strains have been satisfactorily eliminated, bony reconstruction of the fracture can take place. The observation that despite greater distraction the fracture ends eventually unite, has been the subject of extensive investigations concerning chiefly the cause of this process of unification (Fig. 5).

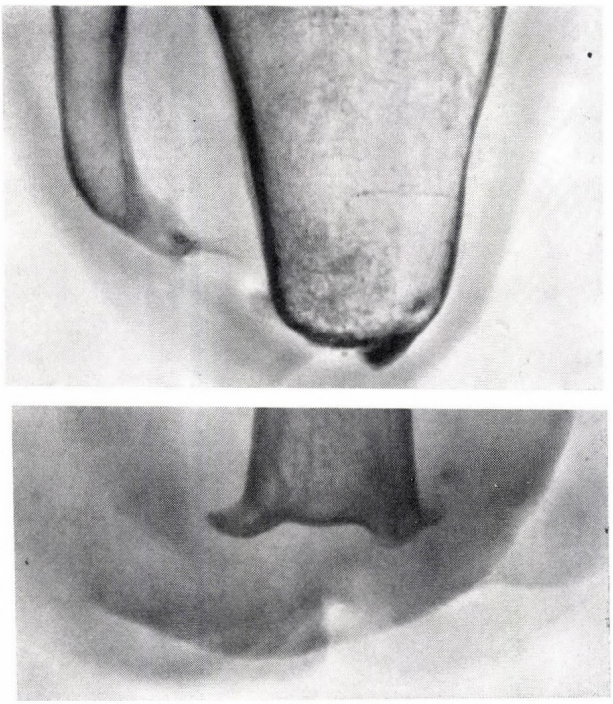

FIG. 8. Formation of osteophytes on amputation stumps

Fractures of the shaft of the femur can heal without marked prolongation of the healing time if displacement exceeding the breadth of the shaft is present. Most comminuted fractures are prone to heal excellently, even if no measures are taken to promote osteosynthesis (Fig. 6).

We have been able to show in previous experiments on animals that good healing tendency of comminuted fractures is due not so much to particularly strong biological regenerative forces aroused there, but rather to the fact

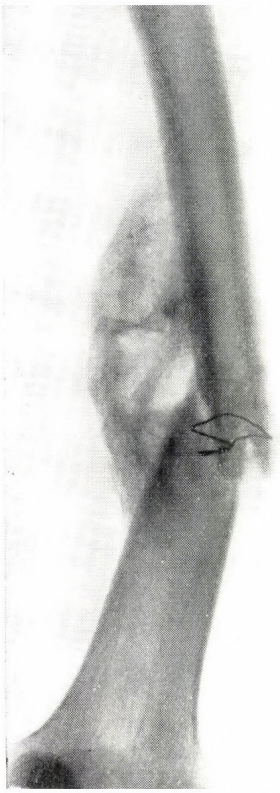

FIG. 9. Callus formed after shaft fracture with strong axial bending

that mechanical disturbing forces (i.e. bending and shearing strains which can be present, e.g. at the fracture site during prolonged distraction) are divided and thus, become uneffective. According to Koch, the way of detachment of the periosteum at the fracture is exclusively responsible for the shape of the developing callus. Pauwels on the other hand, tried to explain the shape of the callus by hydrostatical law (Fig. 7). Bier claimed that a stimulus determining shape starting from the injured organ is essentially responsible for the development of the true regenerate. This concept of Bier seems to be supported by the following points:

1. When the fragments are displaced, callus formation, irrespective of the direction of displacement, takes place always in the shortest way;
2. Formation of osteophytes on the amputation stumps never occurs in axial direction, but always either in the direction of that bone stump from which they have set out, or—in case of amputation of a two-boned limb—in the direction of the stump of the adjacent bone (Fig. 8);
3. In cases of experimental fractures with axial deviation, the callus bridge develops mainly just as it is seen in clinical practice, always on the inner side of the angle (Figs 9 and 10). If during healing the fracture is bent to the opposite side, callus formation present on the outer side of the angle immediately subsides and a new callus bridge forms in the inner side of the new angle;
4. In small displacements *ad latus*, in the angle formed by the fracture ends, a callus bridge develops which will later lead to complete bony union of the fragment ends (Fig. 11);
5. Removal of splinter, e.g. from the tibia, brings about an almost ideal regenerate provided we are not tempted to suture the periosteum to the site of bone removal (Fig. 12);

My collaborator, E. Knöfler carrying out very difficult experiments, made attempts to clarify the conditions present in tissue explants. Without wishing to forestall the publication of his results I have to say that he found that the direction of growth of the fibroblasts is connected with the direction of strain present in the nutrient medium or fracture haematoma. So Knöfler could give a simple physical explanation to this question without presupposing a special 'tissue feeling' (Figs 13 and 14).

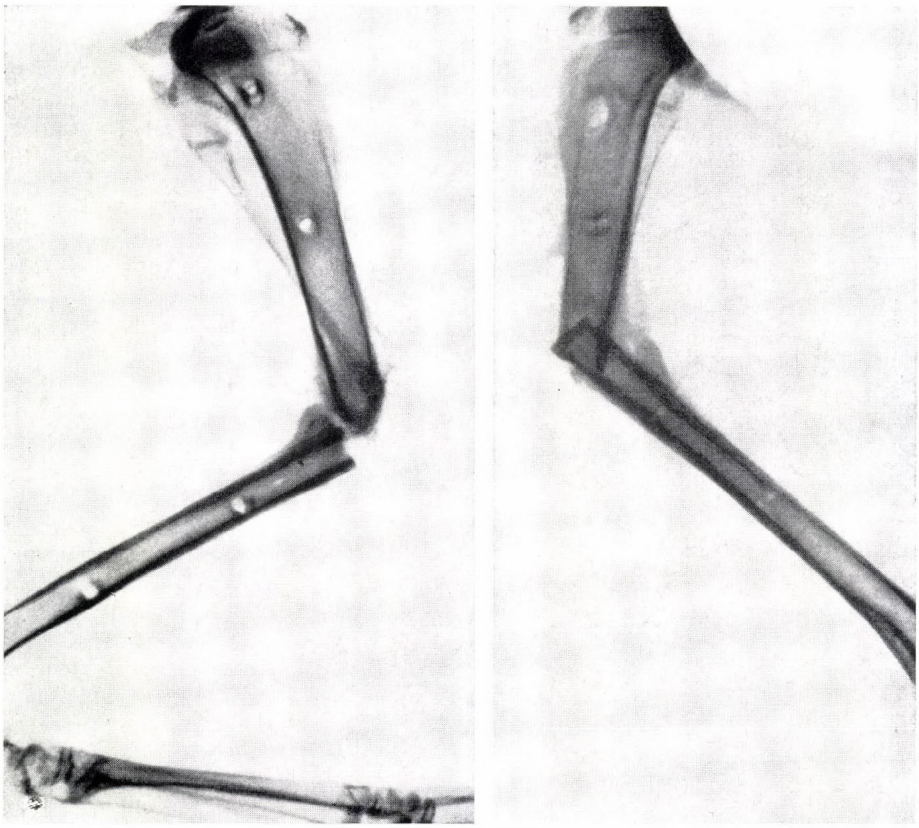

FIG. 10. Callus formed after experimental shaft fracture with strong axial bending in rabbit

I should not like to be misunderstood regarding the importance of accurate reduction and immobilization when I say that the bone ends will eventually unite and often fracture healing does occur undisturbedly even when immobilization had not been quite ideal. I do not think so but I believe that neither the requirements of apposition nor those of immobilization should be exaggerated. Undoubtedly, unsatisfactory reposition and incomplete immobilization are, additionally to deficiences of operative fracture treatment, the main causes of the undeniable increase of the incidence of pseudarthrosis. We have to ponder over each case whether 'ideal' reduction and 'absolute' immobilization can be achieved by the required means, and whether the risk run by operative intervention is reasonably proportional to the aim to be attained. In certain cases we have to make a compromise between X-ray cosmetic requirements and between necessary and accessible ways. Despite our pride felt at thinking of the possibilities afforded by modern techniques of osteosynthesis, we should keep in mind that conservative fracture treatment in the hand of an experienced and, first of all, patient

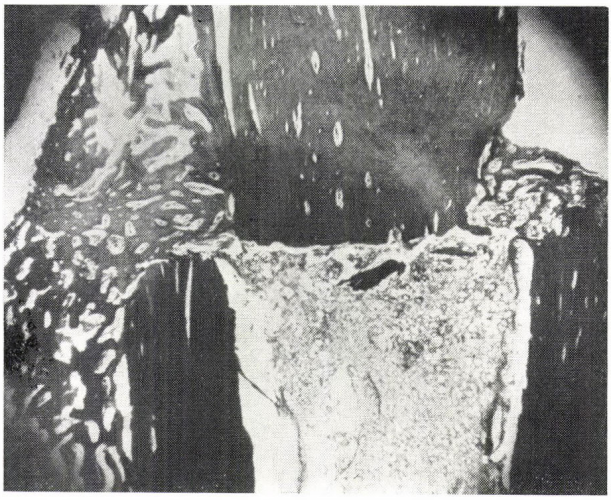

FIG. 11. Callus bridge in the angle area in fracture with displacement *ad latus*

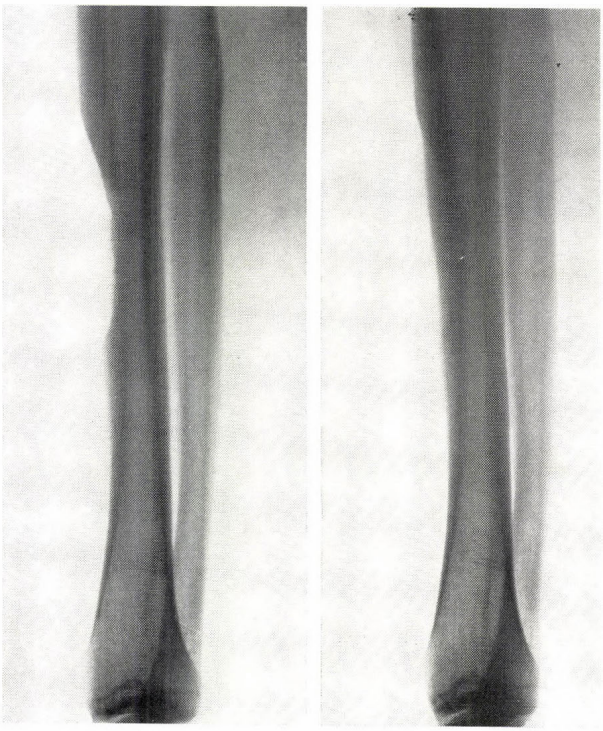

FIG. 12. Tibial regeneration after removal of splinter

surgeon can bring excellent results in the majority of the cases. It is the merit of Böhler that he has shown this again and again.

There is scarcely any vitamin, mineral salt or glandular extract that had not been tested in the last decades as to their negative or positive effect on fracture healing. The results of the investigations are, however, far from being concordant. It seems to be established that Vitamin C deficiency with concomitant deficiency of mineral salts can delay or inhibit repair of fractures. Concerning Vitamin A, B or D deficiency, having likewise a delaying effect on bone regeneration, opinions are divided. Excessive vitamin administration to healthy individuals can by no means shorten the duration of healing. Overdosage of Vitamin D is dangerous as it causes impairment of the vascular walls. This must be emphasized since recently, Crone-Münzebrock, Zaffaroni and others have advocated the positive effect of Vitamin D administration.

Nakamura, Tanabe and others have thoroughly examined the behaviour of various

glands and gland systems during fracture healing. Other authors made efforts to influence the process of fracture repair by giving various hormones to the patient. It is well known from clinical observation that the healing process of diabetic patients is often delayed and bone regeneration is difficult in patients with tabes dorsalis. Administration of anabolic hormones having a positive effect on fracture healing is recommended repeatedly. Amante and Bidone speak highly of androstenediodipropionate,a steroid with bisexual effect on fracture repair. Lücker and Schlaaf have found that Methenolon(1-methyl-δandrostene-β-ol-3-on) accelerates the process of repair in castrated animals. Hageman is right to suggest that positive effect can only be exerted in individuals with low hormonal level. Lambrecht and Kludas found placenta extracts to have a promoting effect on fracture healing. Numerous authors as Bätzner, Baumann, Feria, Grosskopf, Heller, Pass, Prager, Bonaccorsi, Eitel and Lexer, Stainlein and Trouchet (cit. by Matzen), as well as Crone-Münzebrock claim that thyroxine administration has a stimulating effect on fracture repair.

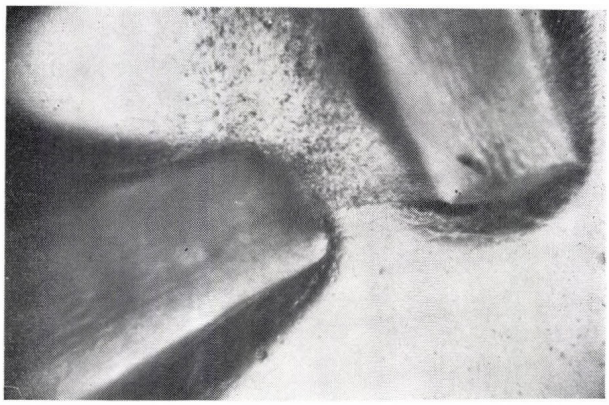

FIG. 13. Cell bundles arranged in the shortest way between the fracture ends (after Knöfler)

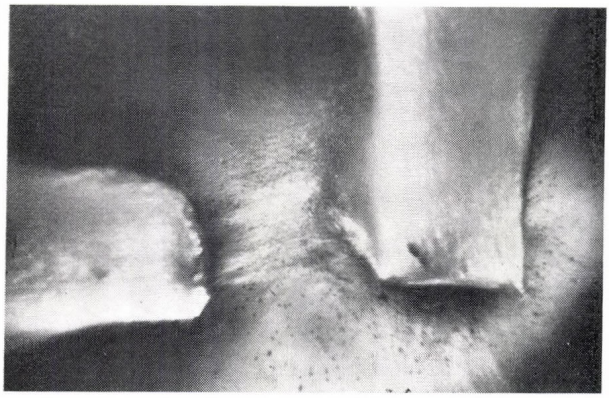

FIG. 14. Examination in polarized light demonstrates that the direction of growth of cell bundles follows the direction of strain of the medium

In experimentally produced factures we could not verify any acceleration of the healing process following the administration of thyroxine or vasodilator drugs or after sympathectomy. In these experiments we have found that as a result of the fracture the vessels have become dilated to a maximum in the fracture area which cannot be enhanced by any medicine or by sympathectomy. In contrast to this, Bernaschek was able to reduce by half or even more the duration of fracture healing by using depot Padutin.

FIG. 15. Defect pseudarthrosis of the ulna treated with wedged graft

In connection with his positive results Bernaschek refers to the work of Bürkle de la Camp and experiments on animals carried out by Frey and Otto. Locally applied lyophilized bladder mucosa was found to have a promoting effect on the course of fracture repair. Mention should be made of the experiments of Welker in which he succeeded in inducing heterotophic bone formation by implanting free grafts of bladder mucosa in the muscle. At the critical evaluation of the works dealing precisely with this subject, one must not forget how difficult it is to judge the effectiveness of any material on callus development. We wish to recall here the statement of Kirschner which, naturally, applies not only to others, that every researcher becomes fond of the subject of his investigation as of his own child and is prone to attribute to it such properties and capacities which in reality only exist partly or not at all. The most important statements from a clinical point of view seem to me as follows: a great variety of disturbances, either those of mineral metabolism or those of hormone and water households, can exert a delaying effect on fracture healing; up to the present there is no certain method to accelerate the course of fracture healing in healthy individuals either by supplemental nutrition or by giving some kinds of materials; in case of disturbances, mentioned above, a substitutional therapy must be instituted. It is an important and much debated question whether medicines can disturb the course of fracture healing. The frequently asserted disturbances of fracture healing and survival of grafts due to antibio-

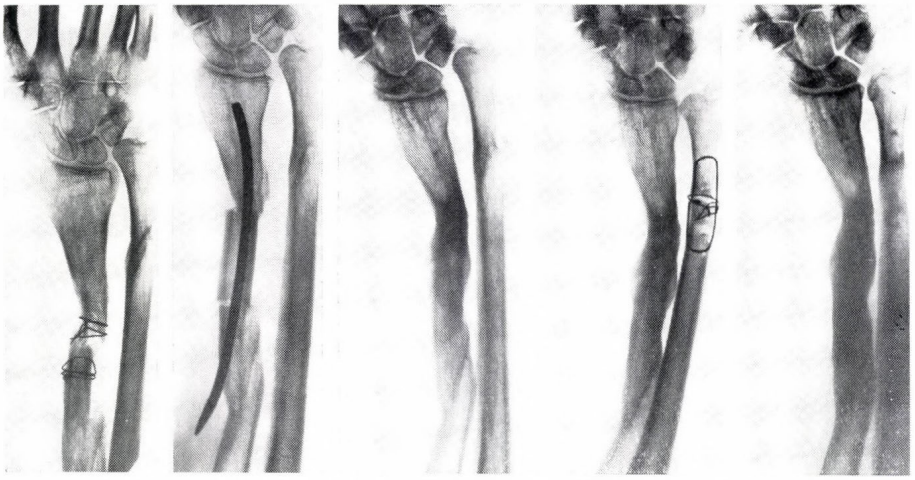

FIG. 16. Pseudarthrosis after fragmentary fracture of the radius treated by stringing method, according to Wanke Griessmann

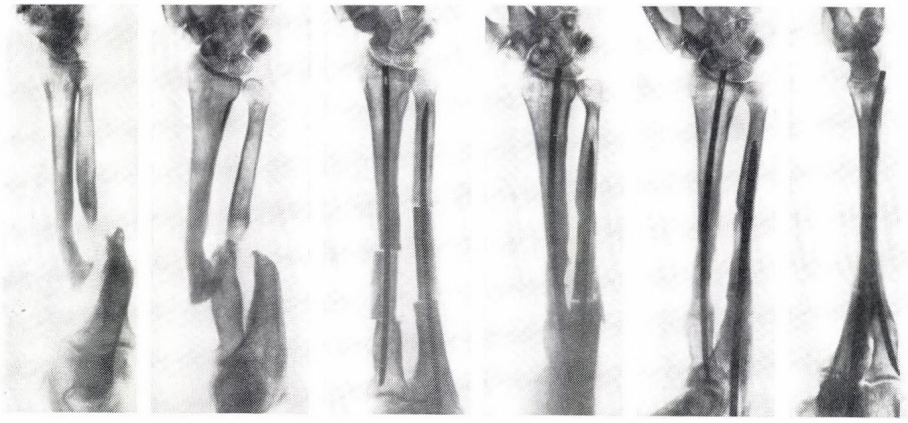

FIG. 17. Defect pseudarthrosis of both fore-arm bones after shot fracture treated by stringing method (fibula covered by autologous periosteum), and 3 and 5 years after fracture

tics have been recently questioned by Lentz and others and in fact do not seem to be verified. Hartenbach believes that cortisone is, at least partially, responsible for the process of differentiation. Overdosage can delay calcification. Cytostatics have been found to inhibit bone regeneration.

High X-ray doses have a damaging effect on callus development and the stimulus of X-ray irradiation cannot accelerate fracture repair, as has been again demonstrated lately by Graggion and Ravazzolo.

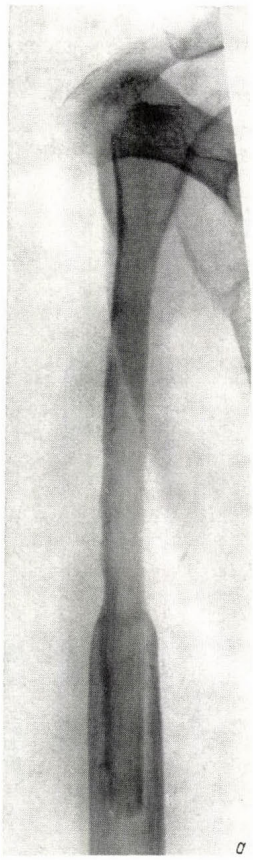

Fig. 18a. Replacement of humerus by autologous fibula graft. Case 1

According to the tasks given to the graft in bone transplantation, the following possibilities are offered:

1. Bridging over of a bone defect by a free transplant;
2. Grafting in order to stimulate bone regeneration;
3. Grafting performed merely to fix the fragments.

When bridging of a defect is performed, both the graft itself and the grafting bed have to meet the highest biological requirements, namely, the larger the defect and worse the grafting bed, the more difficult the bridging over of the defect (Figs 15, 16 and 17). Thus, bridging over defects in the metaphyseal spongious bone segments of the extremities and in spondyloses in the area of vertebral arches is easier to perform than in the diaphysis. In fact, only autologous grafts covered with periosteum proved to be suitable for bridging over of large diaphyseal defects (Figs 18, 19 and 20). Occasional results with homologous material obtained by us, too, especially in younger patients, only proves the rule (Fig. 21).

In some cases homologous and autologous bone material can be employed together, as the homologous bone would mainly undertake the function of support, while the autologous material would meet the biological requirements. The use of homologous half-joint taken from fresh corpses, as suggested by Lexer, proved to be inadequate for arthroplasty, because in a few years, almost regularly, resorption of the graft and stiffening again of the joint occurred. Recently, Imamaliev reported good results in homologous transplantations using half-joints kept previously at $-80°$ to $-150°C$. My collaborator Fleissner could not confirm the optimistic evaluation of Imamaliev in experiments on animals. Whether and how far lyophilized grafts are appropriate for bridging over diaphyseal defects I cannot tell

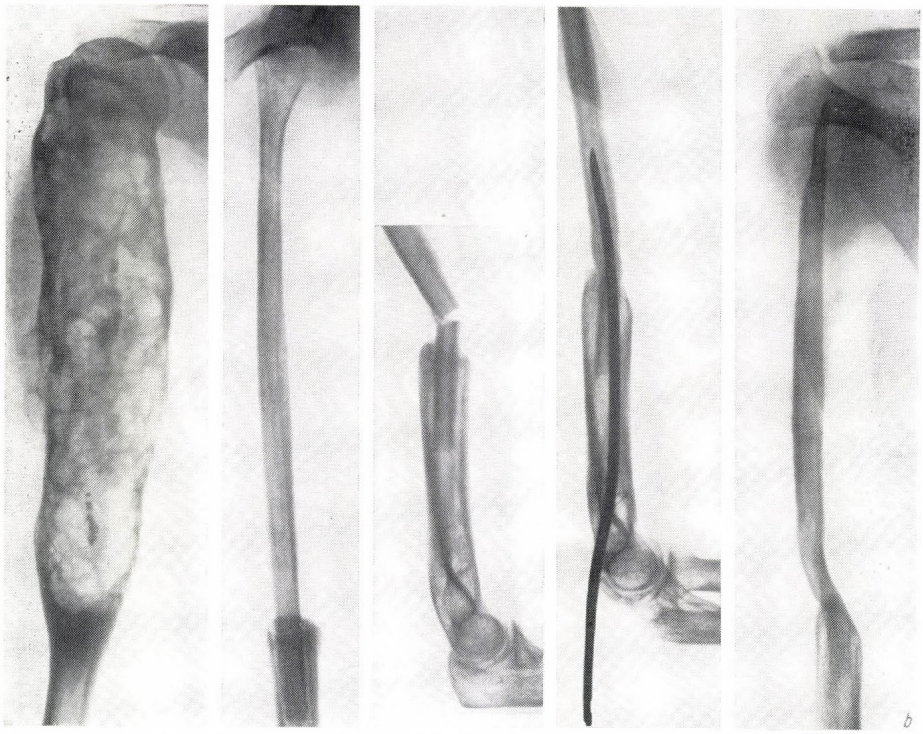

FIG. 18b. Replacement of humerus by autologous fibula graft. Case 2

from my own experience. At any rate, the slighter mechanical strength of these grafts compared with deep frozen ones has to be taken into account with the operation technique (Fig. 22).

For onlay grafts, e.g. with pseudarthrosis, and for spongious filling material with bridging over bone cavities and stiffening of the spine, satisfactory results have been obtained by us for years, using almost exclusively homologous bone material frozen and kept at —30 °C for onlay grafts, e.g. with pseudarthrosis and for spongious material to fill up bone cavities, as well as for stiffening of the spine in operative treatment of scoliosis (Figs 23a and 23b). For fixation of osteotomies, deep frozen and adequately prepared grafts from bone bank proved to be most suitable as screwing material, external splint or even as a kind of medullary peg in hundreds of cases treated in our Clinic (Figs 24a, b and c). It is advantageous for the patient that by using this osteosynthetic material no second operation is needed. Its disadvantage, however, is that no true osteosynthesis can be achieved with it as it is possible with medullary nailing. The most we can attain is early mobility, but no early weight bearing of the limb.

The results of transplantation are greatly dependent also on careful operative technique which causes no more damage to the graft bed as it is inevit-

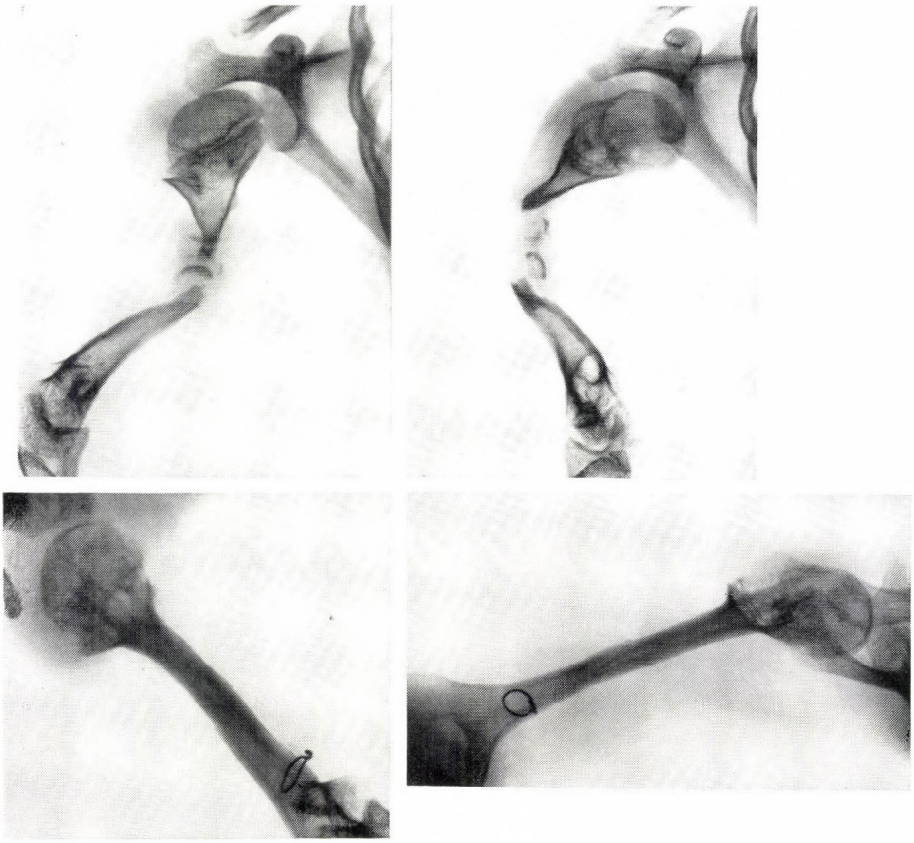

FIG. 19. Diaphysis replacement of the humerus by autologous fibula graft

able. Lexer's requirement that the graft must be inserted as a carpenter would do it and kept in place securely, guarded completely against mechanical injuries by external or internal fixation is valid just as before. In addition, it should be pointed out that the material used for osteosynthesis must be histologically compatible and resistant to corrosion. In opposition to circumstances present in the treatment of fresh fractures, persistent gaps between the graft bed and the graft almost always lead to failure of the operation, since here the forces of regeneration are not sufficient to bridge over even small gaps. It can be stated that the time of healing of transplants given in the literature is usually too short. If the healing time of a diaphyseal transplant is given by an author as 8 to 12 weeks, we must regard such a report with the greatest scepticism.

The mechanism of fracture healing is by no means clarified in its details. For the clinician it is important to have some knowledge of the circumstances that may delay the process of healing of a bone wound. In my opinion, the

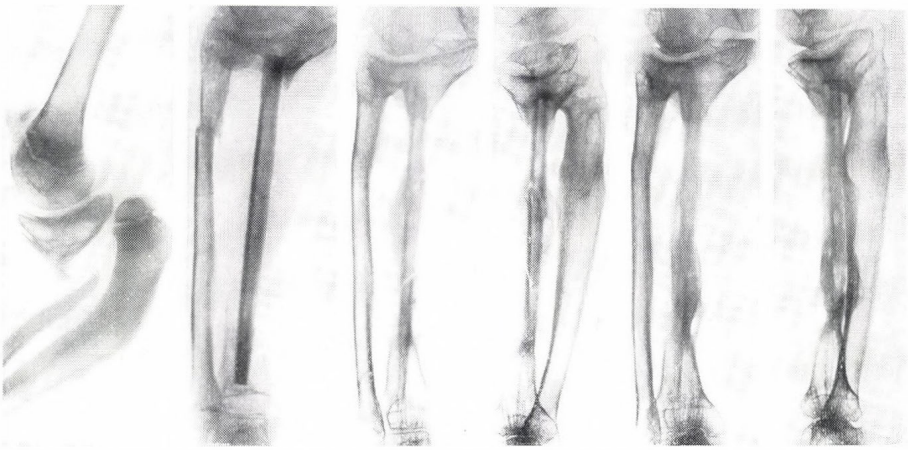

FIG. 20. Replacement of tibia by homologous fibula fragment

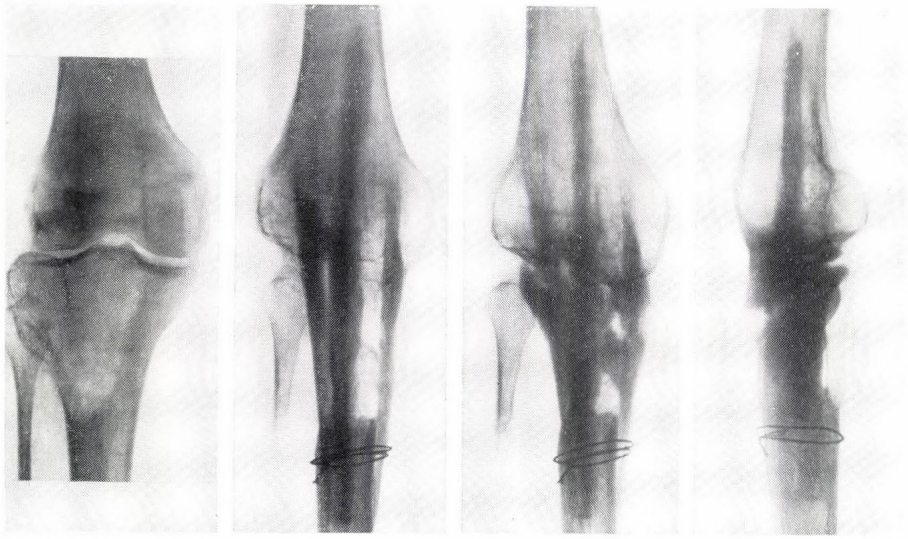

FIG. 21. Giant cell tumour of the tibia head. The proximal part of tibia was resected and the defect bridged over by autologous fibula and 3 homologous grafts from bone bank. The gap between the bones was filled with spongious bone (bank)

main task of the clinician is to protect the bone wound from all those circumstances known from clinical practice and experimental observation that may disturb the course of repair. By the way, we may be content with that statement that a bone wound becomes healed even without our assistance and that our task consists essentially in taking care that this healing

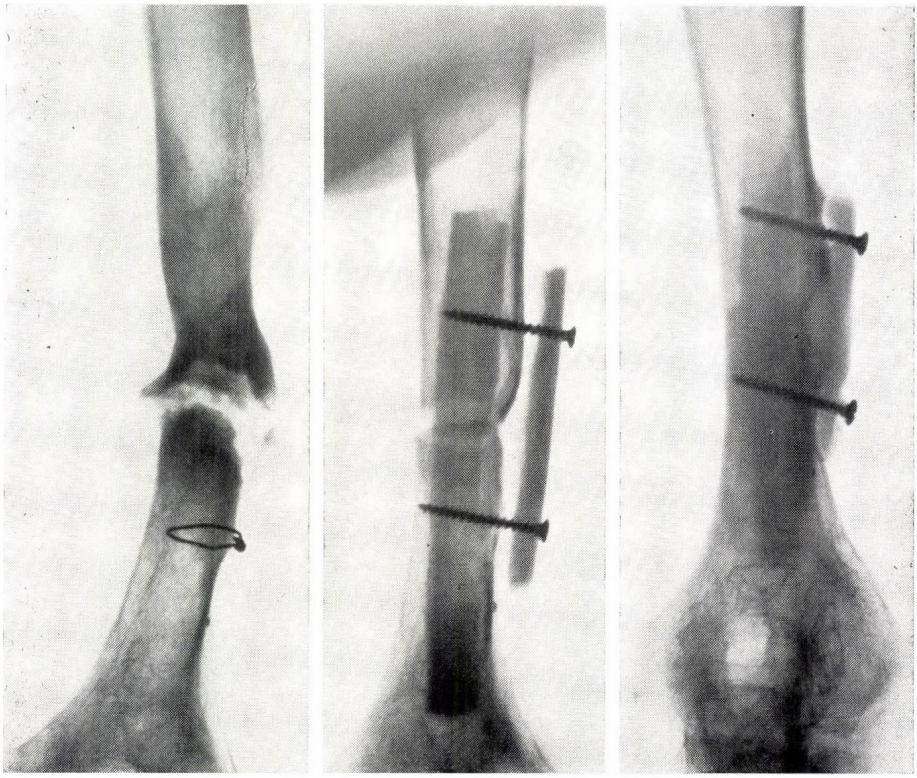

FIG. 22. Pseudarthrosis of the distal part of the humerus bridged over by freshening the bone ends and employing bone bolt and onlay graft. For bolting and onlay graft, homologous deep frozen splinters were used from bone bank

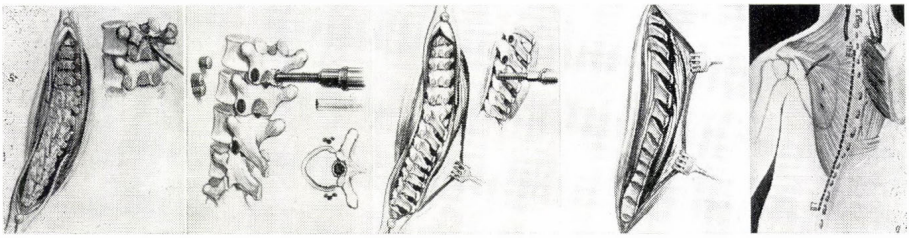

FIG. 23a. Operative technique for scoliosis used in our Clinic

might take place in a situation favourable for later function. Numerous possibilities can disturb the course of healing. However, so far I have not seen the possibility to shorten the duration of healing beyond physiological

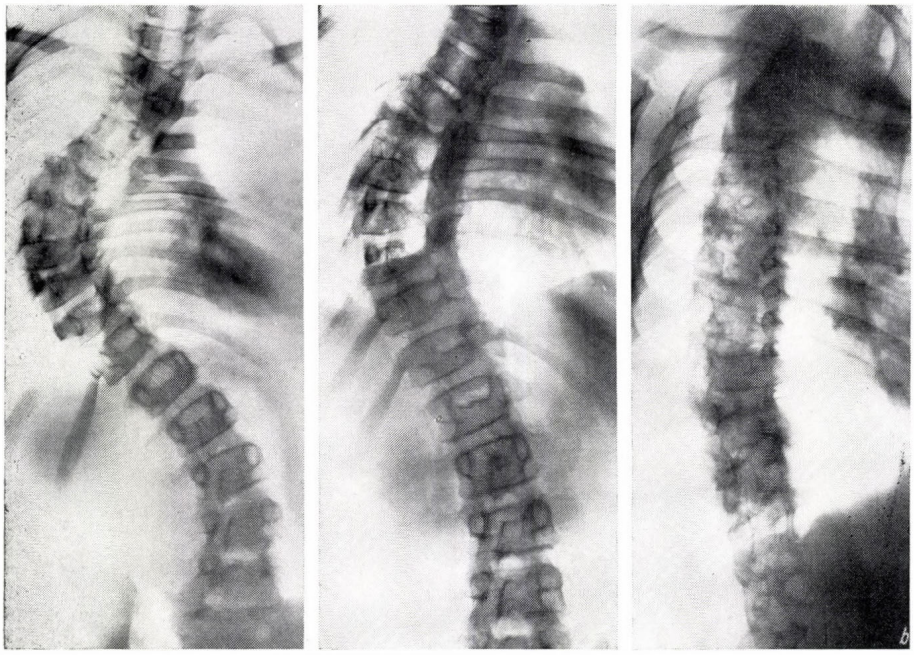

FIG. 23b. Case of scoliosis operation. The time needed for resorption and organization of these homologous transplants—in contrast to autologous material—is longer

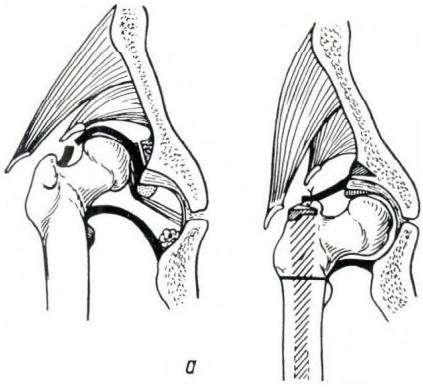

FIG. 24a. Our technique of operative reduction of the hip, plastic operation of the roof of acetabulum, and correctional osteotomy of the proximal part of the femur

limits. Thinking of the great number of open questions we can be consoled by the somewhat resigned verse of Christian Morgenstern:

"Es frisst im Weisheitsfuttersack
Ein jeglich Maul ein Weilchen
Doch nie erreichst oh Schabernack
Die letzten Bodenteilchen."

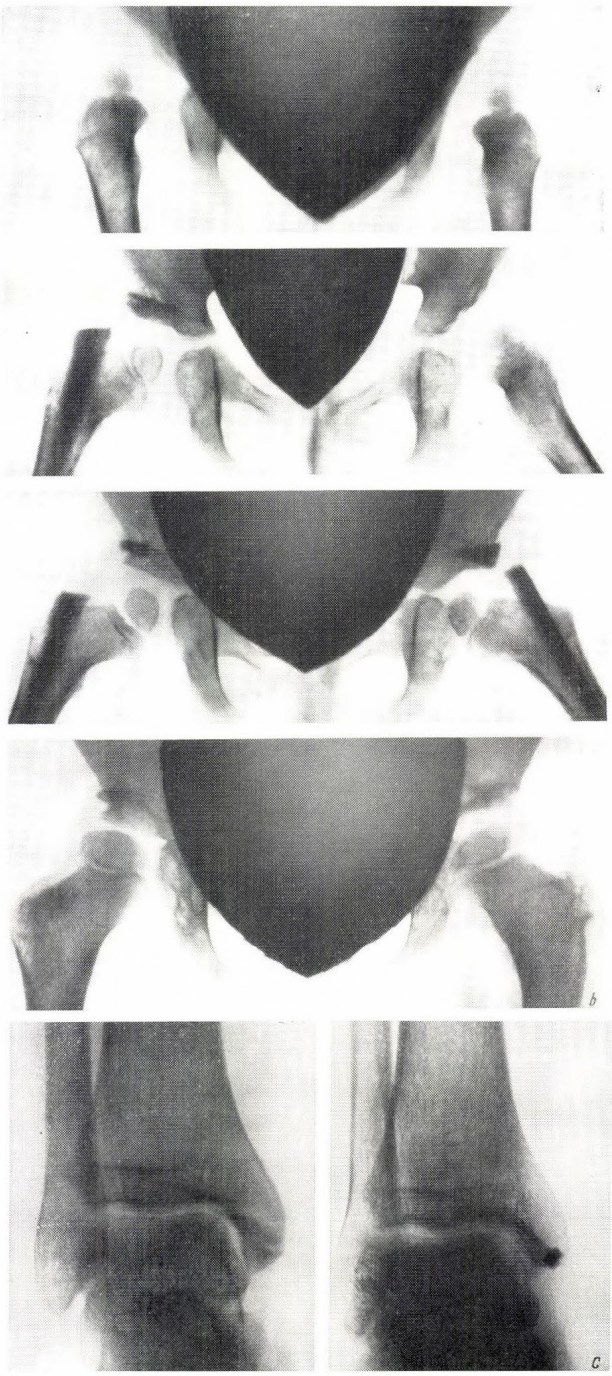

FIG. 24b. Example of operative reduction of the hip

FIG. 24c. Malleolar fracture fixed by homologous bone screw

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Symp. Biol. Hung. 7, pp. 133—135 (1967)

PHYSICAL PHENOMENA AT THE COMPRESSION OF BONES

by

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Clinical observations have shown that compressed cancellous surfaces are united more rapidly and reliably under compression than with simple immobilization. The method of compression has, therefore, been employed also in fractures where instead of cancellous surfaces compact cortical surfaces have to unite. As regards mechanism, according to some authors, all that is achieved by compression is precise and safe immobilization so that its action is purely mechanical, whereas others maintain that compression promotes osteogenesis. The aim of our experiments was to furnish data to decide this problem and to find the physical explanation or, at least, a physical parallel of this phenomenon.

Basset and Becker (1962) demonstrated that electric potential was generated when bones were bent: it was negative on the concave and positive on the convex side. This would mean that negative electricity arises where compressive, and positive electricity, where tractive forces are predominant. This observation encouraged us to study physical phenomena observable at the compression of bones. The Hungarian Bureau of Standards made a repetition of Basset and Becker's experiment possible. After removing the tibia of rabbits, we mounted on it two silver-chloride electrodes opposite each other and measured the potential difference between them by an oscillatory-capacitor electrometer of large input resistance (10^{12}—10^{4} Ω), type ORION-KETI 2517M.

Negative potential was found to have arisen on the concave side of the bent piece of bone, the magnitude of which was directly related to the extent of deformation. Bending in the opposite direction with unchanged electrode arrangement, resulted in a potential of opposite polarity. The magnitude of the potential difference amounted to about 500 μV.

In the second stage of our work, we tried to find a phenomenon resembling the one described above in the physics of crystals. When loading common rock salt crystals asymmetrically, Fischbach and Nowick (1958) observed a phenomenon similar to that described by Basset and Becker that a potential difference arose between the compressed crystal surfaces.

Since these authors observed the phenomenon while studying the Gyulai-Hartly effect (1928a, b) it was natural for us to wonder whether this effect might also be observable on bone specimens. Gyulai found that unilateral pressure caused a sudden rise in the electric conduction of crystals. The

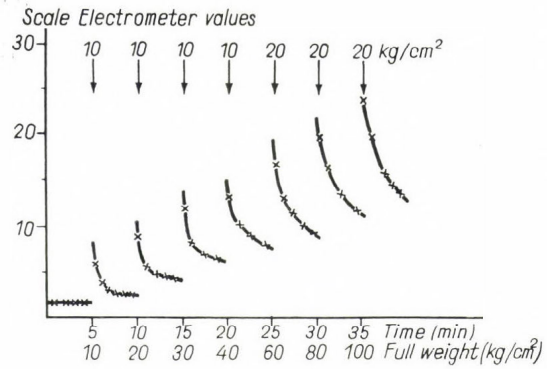

FIG. 1

rise occurs at the moment of loading, and it takes about five minutes for the conduction to regain its original value. Such rises occur again if the pressure is repeated, provided each load is greater than the preceding one. Gyulai and Hartly observed this phenomenon on NaCl monocrystals, whereas the inorganic matter of the bone consists of hydroxyapatite crystals.

Our experiments have shown that the conductivity of the bone specimen also increased when electric current was transmitted and the specimens were compressed. The bone test pieces used by us were parallelepipedal, 2 to 3 mm thick with a surface of about 0·5 cm^2. We used Gyulai-Hartly's compression apparatus and applied 100 to 120 V. The thoroughly dried specimens were statically insulated, and the intensity of the current was measured by a Zeiss projection electrometer. Abrupt pressures of 10, 20, etc. kg/cm^2 caused sudden increases in the conductivity of the samples, but the conduction current decreased after some five minutes. The whole process was a replication of the Gyulai-Hartly experiment (Fig. 1).

In the third phase of our experiments we tried to find other similar features of bone and a crystal system. It was logical to presume that bone behaves as a specially arranged group of crystals in other respects, too. It is well-known that exposed to pressure, alkalihaloid powders recrystallize so that it is possible to compress the finely pulverized crystalline matter into pills, so-called disks. The consistency of the disks is due to a coalescence of the compressed minute crystallites, and a microscopic inspection shows that they are larger after compression than in the original powder. Dried and pulverized bone could likewise be compressed in our experiments into disks, and it recrystallizes quite as readily as powdered alkalihaloids.

The possibility of disk formation reveals another aspect of compression. The fact that pulverized bone which has lost most of its organic substances during long storage is capable of being cemented into a solid block under pressure shows that compression causes recrystallization, i.e. that the crystallites, building stones of the bone are more rapidly remodelled and enlarged if pressed together.

In 1934, one of the present authors demonstrated the Gyulai-Hartly effect on alkalihaloid disks. The effect could readily be demonstrated by us on compressed bone disks, too. It was found, however, that changes in the specific conductivity of the bones and bone disks were 5 to 10 times larger than the values noted by Gyulai and Boros (1940) in connection with potassium-bromide and common-salt crystals.

CONCLUSIONS

From the above experiments we can rightly suppose that compression promotes the healing of fractures at least in two ways. It seems justified to assume that the arising electric field influences the migration of the ions of the inorganic bony matter in a favourable sense, thus, accelerating its development. Compression, applied to crystal nuclei and crystals in formation, plays a similar role. Investigations for a further confirmation of these assumptions are in progress.

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Symp. Biol. Hung., 7, pp. 137–142 (1967)

SEGMENT-PATHOLOGICAL CHANGES IN CALLUS FORMATION

by

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The theories of callus formation are based on well founded experiments and facts. The best known theories concerning callus formation are based on compression (Krompecher 1937, Roux 1895), immobilization (Watson-Jones 1943) and sterile inflammation (Küntscher 1950). Optimal blood circulation proved to be of essential importance in callus formation.

These theories—though apparently divergent—are all valid, though in a segmental sense. By the term 'segmental' we mean that each segment of the extremity has its own biological milieu, not only the traditional proximal, middle and distal thirds, but also the smaller segments, e.g. the upper third of the tibia can be subdivided in three minor segments (Fig. 1). A fracture of the most *proximal segment* is generally an articular fracture as well. In such cases attention should be paid to the involvement of the epiphyseal cartilage which has a special importance in the young organism. In fractures of the *middle segment* the branching of the artery should be taken into consideration since the rupture of the artery may cause an unsatisfactory callus formation in spite of the presence of the spongious bone and thick muscles. The *lowest segment* of the upper third of the tibia is the transition zone between the spongious and compact bone. Fracture healing in this part is generally good, but this is the site of preference of latent fractures.

As regards *joints* in callus formation, one must consider not only the cartilage surfaces, bones and ligaments, but also the surrounding tissues, i.e. muscles, vessels, nerves, and the function of the joint and its metabolism. Taking into consideration all these aspects, the term 'arthron' has been given to the joint (Fig. 2). In a thorough study of callus formation we have to investigate not only the physical, chemical and histological characteristics, but also the biological ones. This conception was the practical ground of introducing our 'directed active movement therapy' in the treatment of certain fractures.

As a result of numerous clinical observations, we may conclude that every segment has its own biological forces which dominate regular callus formation and determine the ultimate fate of the fracture.

The biological conditions stimulating callus formation are as follows: optimal blood circulation, the action of muscles and the so-called osteotropism (Fig. 3a and b; Pap 1941). This latter biological force is not fully

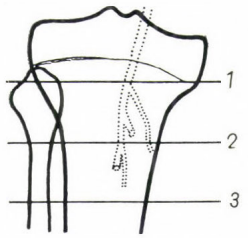


FIG. 1. Segments of the upper third of the tibia

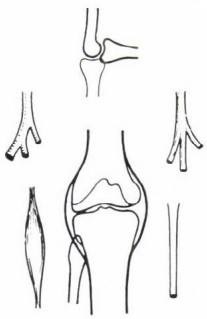


FIG. 2. Scheme of the 'arthron'

understood so far. It is similar to neurotropism and it may be a special manifestation of the regeneration of the living bone segment. The fracture ends which have been greatly dislocated, will find each other and consolidate (Figs 3 to 5). It is known that callus formation may be influenced by nervous alterations causing either resorption or overproduction of callus. Fracture healing is very good in cases of cerebral palsy and in mentally deficient patients. Probably the effect of Child's gradient has to be considered in fractures. It is known that the fractures of the face, shoulder and pelvis and those of the proximal ends of the extremities generally heal better.

During our studies on callus formation, in the light of segment-pathological variations, we have observed certain conditions inhibiting the normal process of callus formation.

1. *Inner decubitus* is encountered in cases when a fracture end presses the soft tissue from inside, provoking first a block of circulation, then bionecrosis, followed by actual necrosis (Fig. 6).

2. *Local shock* ensues in such segments where, in spite of slightly dislocated fractured ends, the local blood circulation is seriously disturbed. Local schock is characterized by the formation of bladders and a pale cyanotic skin (Figs 7 and 8).

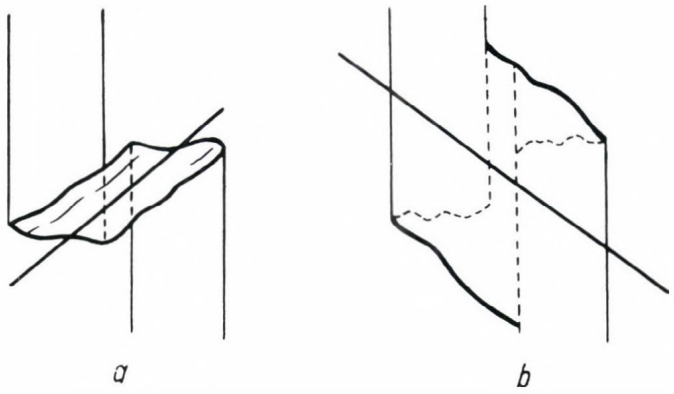
3. *Ischemic contracture* and *myositis ossificans* occur in certain segments, especially in the elbow.

4. *Scarring* is a common complication of fractures. The cicatrizing surrounding tissue may have a retarding effect on fracture healing or it may lead to pseudarthrosis.

5. *Bone necrosis* is encountered according to Böhler in the majority of fractures. But where the segmental muscle mantle is thick and blood circulation is uninhibited, bone necrosis is of no importance, because a muff-like callus is formed and the fracture will consolidate in due time (Fig. 9a, b). Circulation can be stimulated by active motion.

6. Finally, the various *swellings* may be mentioned, i.e. gravitation, inactivity and strangulation oedema. After loosening the pressing plaster cast, active exercises are recommended. The greater the swelling, the more actively should the patient perform the prescribed exercises (Watson-Jones 1943).

As a conclusion we can state that bones are sensitive to circulatory disturbances. This sensitivity is increased in case of fractures. Compression and immobilization are important in the fractures of most segments, but optimal circulation is, in our opinion, the decisive factor. This is confirmed by rib fractures healing very well in spite of permanent breathing movements



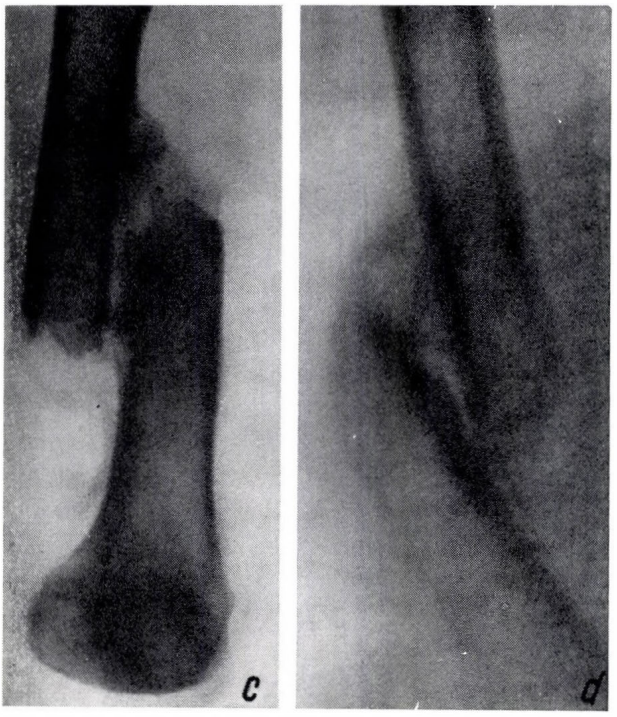
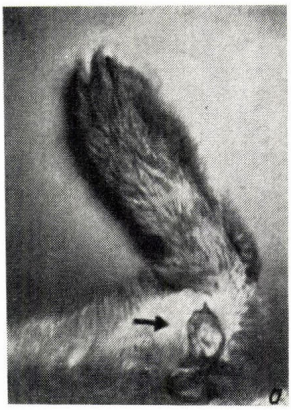


FIG. 3. Scheme of the essence of 'osteotropism' (a and b) Osteotropism in experiment on rabbit (c) and observed in human fractured bone (d)

FIG. 4. Osteotropism manifests itself not in all directions around the fractured ends, but only on the sides of the bones facing each other



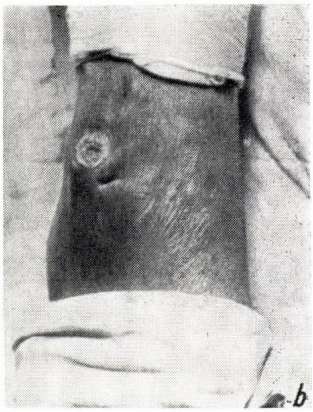


FIG. 6. Inner decubitus caused by the inner presssure of the bone fragment. The soft tissue becomes first ischaemic (a), later necrotic (b), the final result being the non-union of the fracture ends

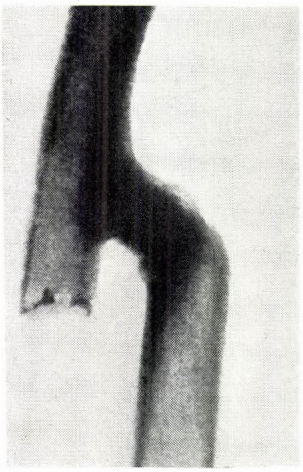


FIG. 5. Healing of a fracture in the lower segment of the femur. Osteotropism is present in this region and, therefore, bony consolidation occurred in spite of the dislocation of the fragments

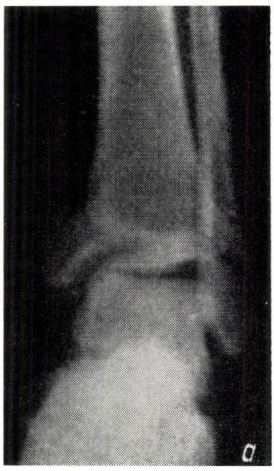


FIG. 7. Local shock in a malleolar fracture with insignificant dislocation (a). The local circulatory disturbance caused bladders (b)

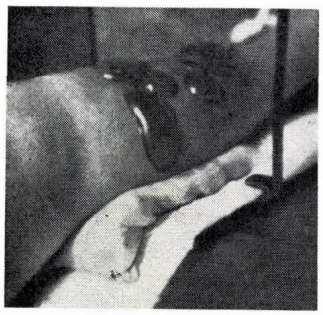


FIG. 8. Local shock of a great extent in a fracture of the upper third of the tibia. Note the large bladders indicating serious local disturbance of circulation

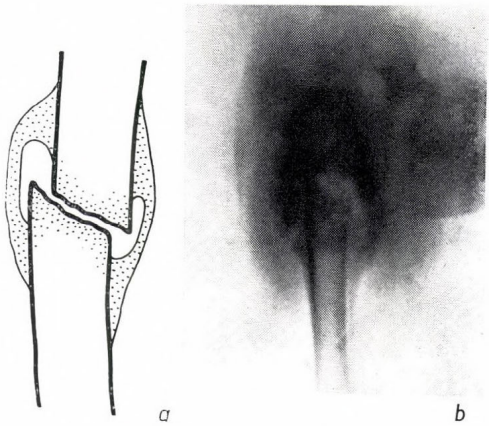


FIG. 9. Scheme of muff-like callus of a fractured bone (Charnley). Periosteal callus may be formed even during continuous motion. After the formation of the periosteal callus, the endosteal and osteal callus will develop undisturbedly. No internal or external fixations are needed (a). Fracture of the femur of a child aged 4 years. After the formation of a muff-like callus, the femur is clinically firm 21 days after the injury. Note the central gap between the ends which will later disappear (b)

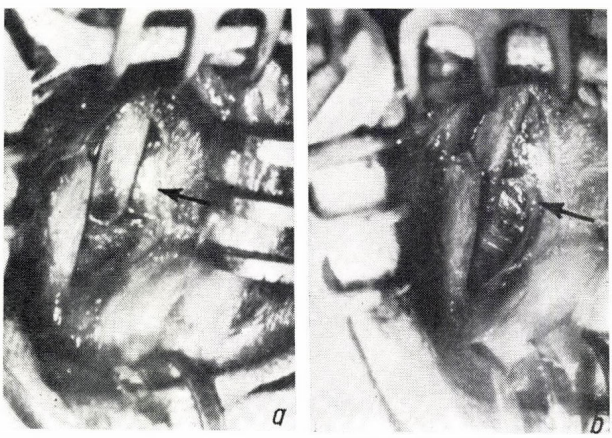
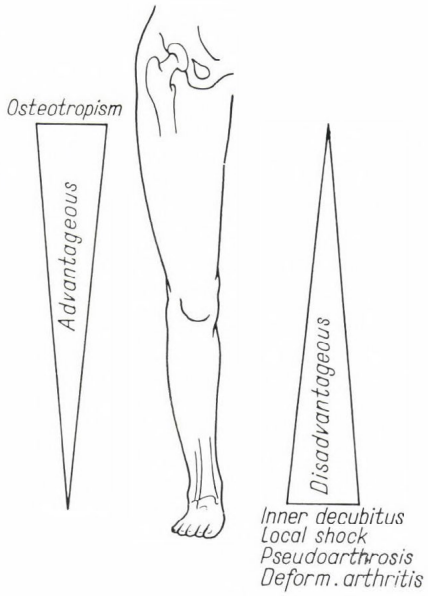


FIG. 10. Experimental oblique fracture on the 8th rib of the rabbit (a). Note the dislocation which occurs with every breathing (b). Nevertheless, fractured ribs are known to consolidate early

(Figs 10 and 11). For detailed segment-pathological description see Pap (1963). In Fig. 12 a scheme is presented on the segment-pathological changes of the lower extremity.

FIG. 11. Histological preparation of a fractured rib (rabbit) 15 days following intervention. Chondral and desmal callus formation as well as new formation of bone (Z. Papp and Szigeti)

FIG. 12. Scheme of the segment-pathological changes of the lower extremity. The effect of biological factors favourably influencing the course of fracture healing are observed in the upper third of the femur. The lower segments of the tibia appear to be more liable to segment pathological changes. The superposition of segment pathological changes may modify or determine the fate of some fractures



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Symp. Biol. Hung. 7, pp. 143–151 (1967)

STRUCTURE-FORMING ROLE OF FUNCTION IN THE HEALING OF FRACTURES

by

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The principal aim of fracture treatment even thousands of years ago was to attain good functional results, because the persons who suffered injury during their struggle for existence wanted to use their injured extremities as soon as possible. It is highly probable that in the group of fractures in which the reduction of function was caused by pain alone, forbearance and treatment lasted only as long as pain was felt. The reduction of fractures with considerable dislocation was also aided by man's aesthetic sense. Setting the injured member in repose decreased the pain, but at the same time atrophied the tissues that had ensured good function. The knowledge of the treatment of fractures during the past centuries has been developed for the purpose of solving the contradiction that the aim of treatment is the restoration of normal function and the movement of the extremity, but the means by which it was done was setting it in repose.

As a branch of modern medical science, traumatology is undergoing a significant development. This important problem, however, is to some extent unsolved even today and, therefore, the attention of research and clinical workers is focussed on questions of detail concerning callus formation and fracture treatment. During the past decades fracture-treatment methods have followed one another; the development and technical perfection of X-ray examinations have permitted a more thorough scientific cognition of details. But, at the same time, they have also resulted in the spreading of the view that a restoration of perfect anatomical form must be aimed at in healing each fracture. A subsequent insufficient function of anatomically well-set fractures has again called attention to the true aim of fracture treatment: the possible best restoration of the *function* of the injured part of the body, and so that of the whole organism. This stimulated the development of various modern fracture treatments, each of which endeavours in its own way to solve the contradiction that the ensuring of tissue repose, necessary for the restoration of the original biological form, or the perfect restoration of the bone structure sometimes impairs the working of the tissues ensuring function to such an extent that normal function cannot be re-established after the restoration of bone continuity.

According to the fundamental principle of one of the fracture-treatment tendencies in practice today, the displaced fracture ends must be fitted together and kept fixed until the bony callus is formed and, while exercising the non-fixed joints continuously, the functioning of the fixed segments

must be restored after removing the plaster. During the application of this treatment method, while waiting for the fractured bones to set under the plaster dressing, such secondary alterations in the fixed extremity sections may ensue which will not disappear altogether and so influence the healing disadvantageously. The adherents of another fracture-treatment tendency do not employ external fixation at all to maintain function, or only fix the fractured bone in such a manner that the advantageous structure-forming effect of function should be able to assert itself everywhere. Others profess to be the followers of the functional view. Their method of treatment is confined only to the bone tissue by bringing about a rigid internal fixation in the fractured bone a bone-unifying operation. This allows the earliest use of the tissues which bring about movement of muscles, ligaments, tendons, etc. and so avoid 'fixation disease' and all its unfavourable consequences. Actually, good function does not cease, and the prospects of maintaining it are favourable.

In the past 25 years we have witnessed a significant development of the bone-unifying surgical procedures. The greatest result, however, is not the diversity of these methods, but the general recognition of the principle that the bone-unifying operation is performed primarily for re-establishing function, while the reconstruction of form is only of secondary importance.

One of the manifestations of functional treatment now prevailing in the treatment of fractures is the directed-movement treatment elaborated in Debrecen. According to our experience, we believe that it is suitable for bringing about bone healing in a certain group of fractures without employing either external or internal fixation, and, in the light of our segment-pathological knowledge, to re-establish proper function. In those types of fractures where the omission of external fixation would involve the danger of the failure of bony unification, it would be advisable, in addition to the acknowledged classic medullary nailing, to follow the bone-unifying operative technique elaborated by the *Arbeitsgemeinschaft für Osteosynthese*. The latter procedure almost immediately gives the injured extremity a possibility of complete function after the reconstruction of bone continuity. Early function ensures healing, prevents 'fixation disease', and by the time the callus appears, complete function can be expected.

The question is which are the clinical observations as a result of which we may rely on the success of the method of treatment, mentioned above, and which direct our attention to the primary role of function in callus formation, too.

1. Favourable biological milieu necessary for callus formation can only be imagined in functioning extremity segments and only in the interest of function. Non-function of parts of the body can only lead to atrophy.

2. Though ideal fitting of fractured bones is most advantageous for the healing of fractures, numerous observations verify that excellent extremity function may be attained even without it, if the treatment can be carried out in the interest of function.

3. Even after ideally fitted and set fractures one may often see the development of the 'fixation disease' and its unfavourable consequences.

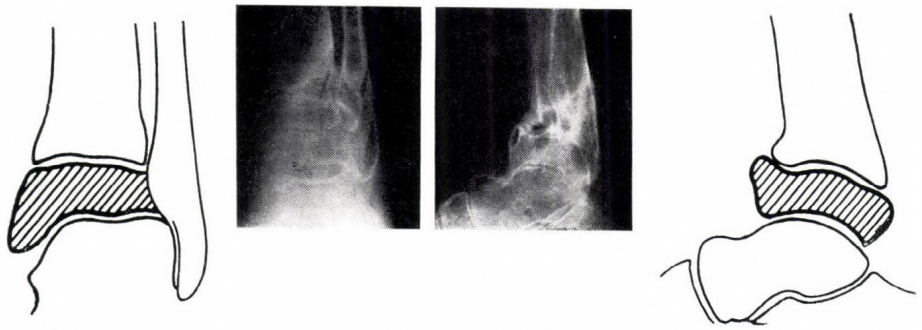


FIG. 1. Supramalleolar tibia pseudoarthrosis. Besides a rigid upper talocrural joint, motion brings about a surface similar to that of the talus

This can be observed mostly in such cases in which external fixation and the lack of function accompanying it has exerted its harmful biological effect for a long time.

4. Clinical practice can provide numerous characteristic examples of the decisive effect of function on the development of structure. Below, a few examples are presented regarding callus formation and bone healing.

(a) It is commonly known that in the case of shin fracture the setting of the bone very often fails to ensue in the tibia, if the early reconstruction of the bone continuity of the fibula results in its propping up. In such cases the biological stimulus so necessary for the setting of bones, the pressure, the muscle tone, etc. manifested during the course of natural function,

FIG. 2. Supracondylar femur pseudoarthrosis. In case of a rigid knee, a structure similar to that of a knee articulation is brought about in the pseudojoint

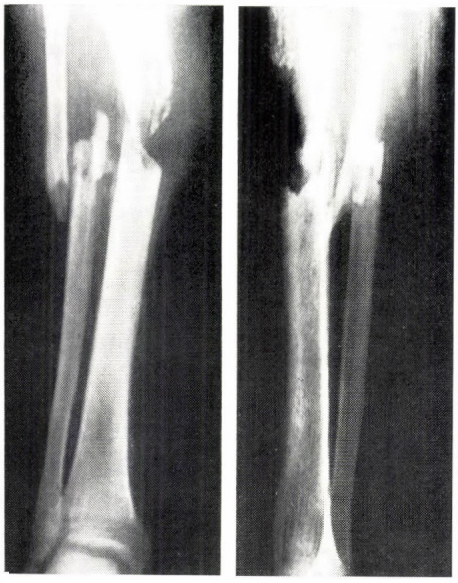


FIG. 3. X-ray picture of an open shinbone fracture with complications

exercise no influence on the broken ends of the tibia. The fracture of the tibia usually sets sufficiently after a fibula ostectomy is performed to avoid a threatening pseudoarthrosis.

(b) During the treatment of fractures adjacent to joints, a considerable limitation of the movements of the joints ensues on account of the lengthy external fixation. If in such cases, in the very interest of the restoration of joint function, the plaster dressing is removed before the complete setting of the callus, and we begin to move the joints hitherto incapable of motion, the organism itself frequently brings about the necessary movements on the given segment of the extremity much easier by loosening the not yet set callus than by loosening the joint tissues that have shrunk as a result of the lengthy fixation. In such cases the provoked function results in such a structure in the callus which is very similar to that of the adjoining articulation, thus, proving that this suits the biomechanical requirements best in the section of the given extremity. A good example of this is the supramalleolar pseudoarthrosis shown in Fig. 1. In such a region the bone continuity is generally easily re-established, yet, on account of the lack of motion of the talocrural articulation, a joint-like structure developed instead of a callus, because the organism attempted to bring

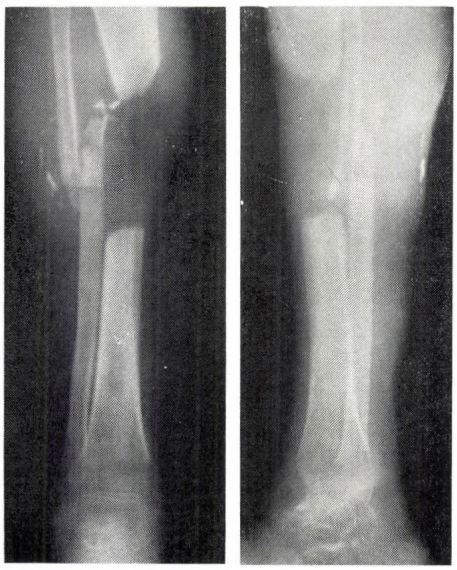


FIG. 4. After removal of the necrotic tibia segment, a pseudoarticulation developed with significant defect

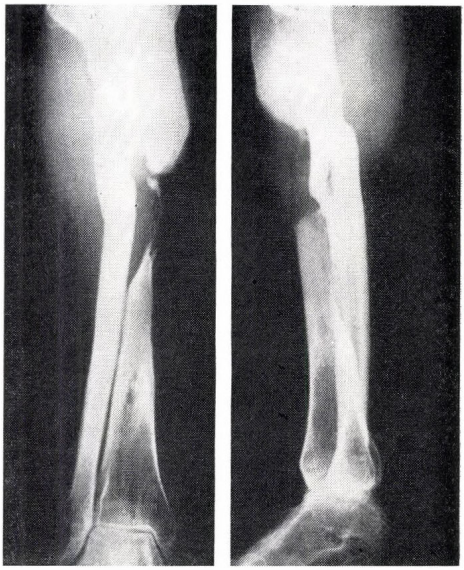


FIG. 5. After shifting the fibula, the bone assuming the function of the tibia has been transformed structurally, too (it has 'tibialized')

about the lacking movements here at this particular section, and this is why the distal fractured segment developed like a talus. Characteristic of the phenomenon, mentioned above, is the second case, too (Fig. 2), where, on account of the treatment complications of the femur fracture, it was necessary to apply a plaster fixation for a rather long time. Before the bony unification of the fracture ends was completed, the fixation was terminated and exercising of the knee movements was started, because it was feared that the movement of the knee joints would be lost for good. This procedure brought about the development of a knee joint-like structure and function.

(c) The structure-transforming capacity of function is best verified by those cases where the replacement of the defects of the tibia was carried out. In these cases it may be observed that as long as there is defect in the tibia, a limited thickening will be brought about on the fibula only at the site subjected to the greatest flexion, because the structure adapts itself to the aphysiological function in this manner. Inasmuch as the fibula is employed for abridging the tibia defect during the course of the operation, and so assigning to it the role of the tibia, in a short time the transplanted, i.e. the shifted, fibula having assumed the function of the tibia is transformed in such a manner as to be able to meet the requirements of the

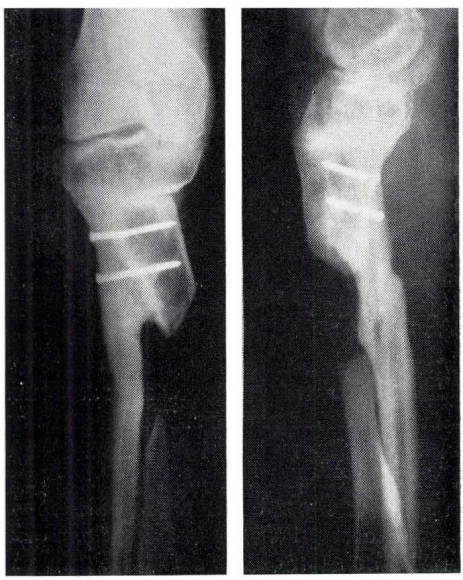


FIG. 6. By osteotomy of the subcondylar tibia, the loading of the extremity was insured in the right direction

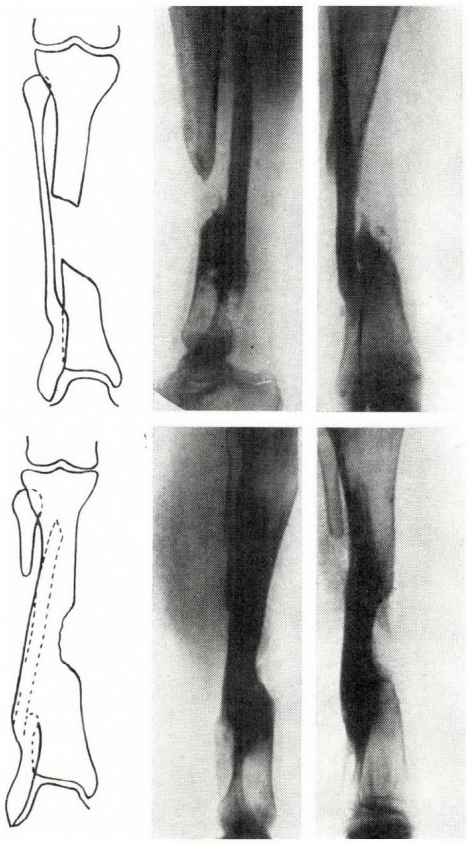


FIG. 7. The fibula substituting the tibia defect first takes over its function, then becomes similar to it in structure, too

altered use. Under the influence of the tibia function, the structure of the bone also becomes similar to it—the fibula 'tibializes'. Those parts of the fibula which do not function, become practically atrophied during this time. The increase or decrease of the function, in other words its changes, alter the structure in its own interest. An example of this is the following case. A 23-year-old man suffered a fracture of the shin as a result of a traffic accident. The open fragmentary fracture became complicated by osteomyelitis (Fig. 3). After the removal of the necrotized bones, a widespread defect of the tibia remained (Fig. 4). The shifting of the fibula restored the continuity of the shin and, under the influence of function, the fibula tibialized (Fig. 5). The axis of the extremity was restored by osteotomy (Fig. 6), whereupon the patient, without resorting to any appliance, regained his ability to walk and his capacity to work.

The following is an example of the structural transformation ensuing as a result of the operation mentioned above; the shifting of the tibia was sufficient to abridge the tibia defect which had developed as a result of osteomyelitis, because the shifted bone portion first took over the function of the tibia and then its form (Fig. 7).

If a graft is placed in the tibia defect, the free graft will get into a much more unfavourable biological milieu than the shifted fibula. The following figures show the results of such an operation.

The open, fragmentary shin fracture of a 24-year-old man (Fig. 8), complicated by osteomyelitis. The broken segment necrotized and a defect remained after its removal (Fig. 9). The defect was replaced by an autoplastic tibia splinter (Fig. 10) 14 months after the injury. A year after the operation, in spite of the fact that the patient was already walking with the help of an appliance and was able to move his joints, no essential change of form was to be seen either on the fibula or the transplant (Fig. 11). Another year

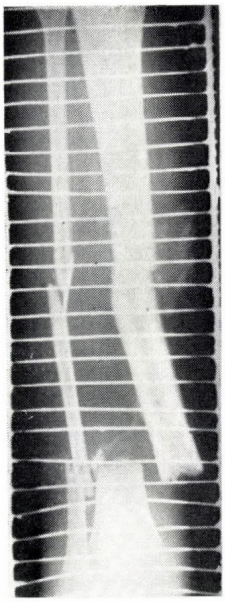
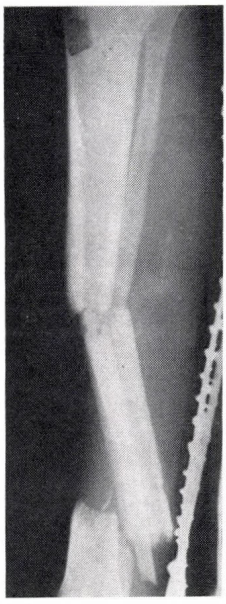


FIG. 8. X-ray picture of an open, fragmentary shin-bone fracture



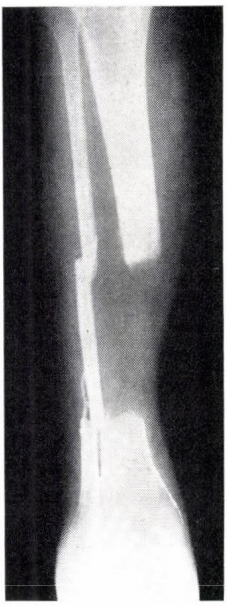
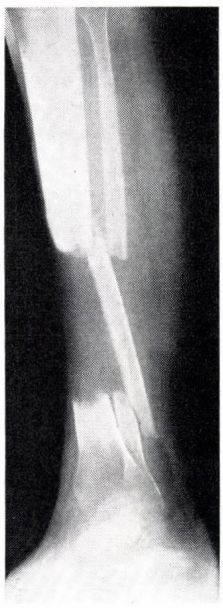


FIG. 9. Defect which developed after the removal of a necrotic bone



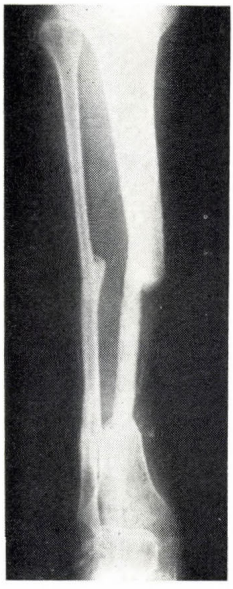
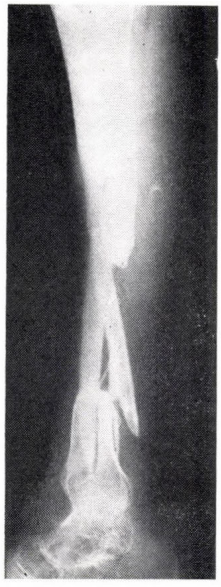


FIG. 10. Autoplastic tibia graft substituting a tibia defect



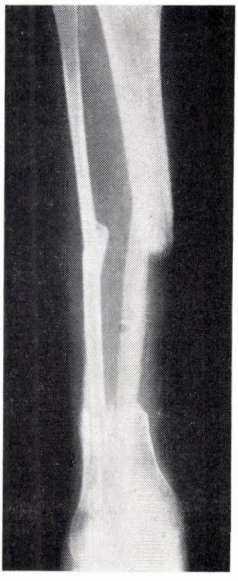


FIG. 11. No significant reconstruction has yet appeared in the transplant one year after transplantation

FIG. 12. Another year later a fatigue fracture can be observed in the transplant

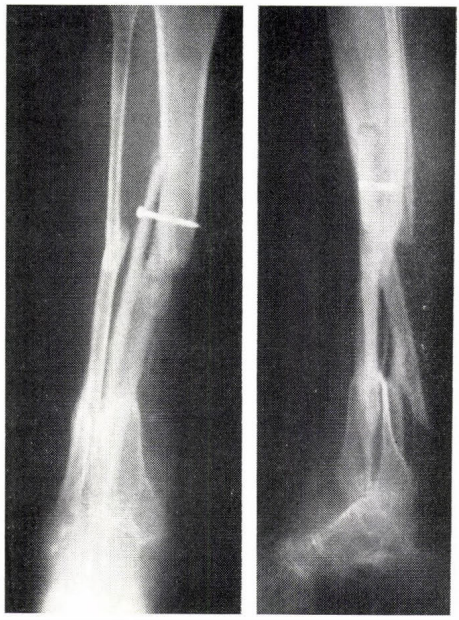


FIG. 13. Two years after another bone transplantation a structure has been produced conforming to the function of the tibia

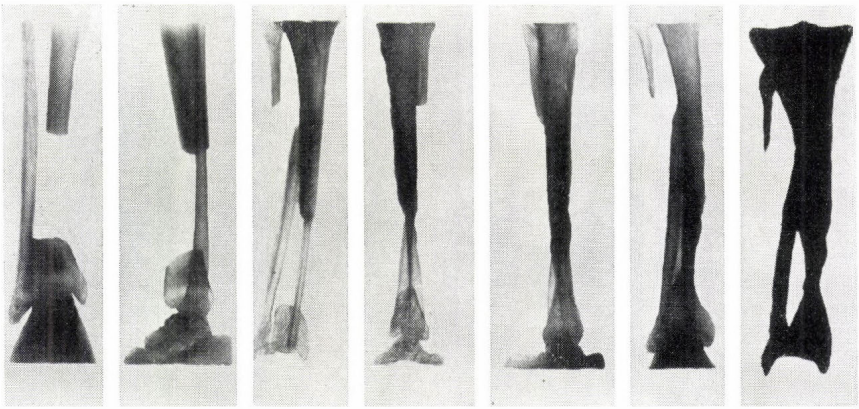


FIG. 14. Supplementing the tibia defect by fibula shifting from the same side and transplantation of a fibula segment from the opposite side

later a fatigue fracture ensued in the graft (Fig. 12). A preserved splinter was then transplanted, but it took two more years for the transplant to develop into a structurally suitable tibia substitute (Fig. 13). By this time the

patient was able to discard his walking appliance and resumed his working as a turner.

In the disease process of the next patient, a simultaneous assertion of the above two viewpoints can be observed (Fig. 14). Into a tibia defect of similar origin as the above, a fibula segment was transplanted from the other side, but at the same time the fibula of the identical side was shifted to the proximal tibia end. Healing followed essentially faster than in the preceding case where the defect was abridged only by the tibia splinter, but a fatigue fracture ensued in the fibula segment also, and the change of structure here, too, was seen to appear later than in the shifted fibula, signifying that the biological conditions for adaptation are much more unfavourable in the case of a free transplant than in that of a shifted fibula retaining its surroundings.

A few characteristic examples have been collected in this report proving that the occurrences of callus formation and bone healing can be properly assessed only on the basis of functional treatment. The observations of the clinician co-operating in the healing of patients and following up the course of their healing stand in harmony with research results, too; all this proves the unity of theory and practice.

Symp. Biol. Hung., 7, pp. 153—158 (1967)

EXPERIMENTAL AND CLINICAL SOLUTION OF THE CALLUS PROBLEM

by

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THE APPARENTLY difficult solution of the callus problem can be simplified by dividing it in two problems: 1. the *formation* of callus, and 2. its *further fate.*

Callus is formed only by *inflammation*, the origin of the inflammation being entirely unimportant. Whether callus is formed owing to chemical, thermic, mechanical, allergic or bacterial stimuli, it displays the same features provided the intensity and duration of inflammation have been identical. By experimentally induced inflammation in the medullary cavity, a desired amount of callus can be produced ranging from the thinnest periosteal layer up to massive sclerosis seen in osteomyelitis. *No callus formation can take place without inflammation.* The callus formed following fracture or osteotomy is of chemical origin, brought about by breakdown products arisen at trauma or operative intervention owing to destruction and death of cells. Extensive callus formation can be induced by a very simple experimental procedure; by introducing a sterile wire (as that used by florists) into the medullary cavity of a long bone. The rusting wire brings about a chemical reaction leading to extensive endosteal and periosteal callus formation (Fig. 1). A fine, periosteal layer induced in the same way is shown in Fig. 2. Here a human fibula is shown in which the newly formed periosteal layer was produced for the purpose of transplantation. In this experiment the callus was protected from mechanical stresses, so that the bone remained entirely intact.

The classic experiment for callus production by pure mechanical effect is Martin's experiment (Fig. 3). Martin, however, did not recognize the mechanical factor in his results. After resection of the radius in the dog, the ulna, which is by far thinner than the radius, was overloaded by mechanical strain due to intermittent bending. By this excessive mechanical employment callus formation occurs displaying the same features as that occurring in the experiment in which iron wire has been employed. Here the way for hypertrophy is open. Krompecher has already demonstrated that even in cases where no such excessive mechanical stresses are present, but loading is within normal limits, fine periosteal bone layer is formed. The often enormous amounts of calluses arising in pseudarthroses have a similar explanation namely, as resulting from mechanical overloading of the bone substance at the fracture ends facing each other. In such cases movement is not divided and attenuated by the interposed cartilage and so *arthritis deformans* may develop, in which the bones lying opposed to each other in the joint are

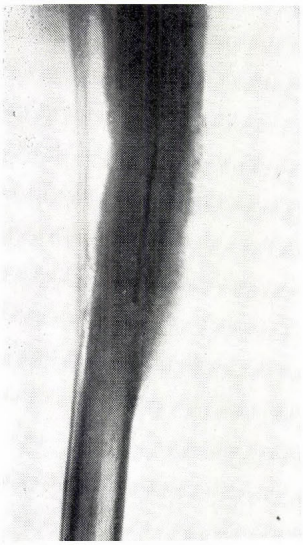


FIG. 1. Extensive endosteal and periosteal callus formation induced by chemical stimulus (rusty wire) in the tibia of the dog

overloaded due to the insufficient function of the cartilage. This leads to formation of marginal condensations which should also be considered as calluses. In arthrosis deformans all the signs of inflammation are demonstrable. All these calluses formed in different ways are similar regarding their histological features as well as their X-ray appearance.

Chronic inflammation may cause enormous thickening of the bone. Figure 4 shows an enormously thickened tibia of the dog, persistent for two years, which was evoked by introducing a piece of sulphur in the medullary cavity. This procedure exerted a *sustaining stimulus* on bone which reacted by strengthening 100 times. Here *function and inflammation* are of about equal value.

We wish to emphasize again that the calluses formed after fracture and osteotomy are of *chemical origin* and so entirely afunctional. The effect of function asserts itself only in calluses formed by chronic influences as in case of pseudarthrosis and hypertrophy mentioned above. (Here again it should be repeated that excessive stimuli lead to inflammation.)

As a result of inflammation, tissular proliferation arises inducing bone in a few days and automatically transforming into bone, provided no mechanical force intervenes by disturbing or destroying it. Here the mechanical factor appears which will determine the further fate of the callus, namely, whether the fracture gap will be bridged over by bone or by pseudarthrosis. However, the young callus is very slightly resistant to mechanical loading. It can be distracted to a very small degree only as it tears apart even if slight tensile, jerking or shearing stresses are applied to it. Compression can destroy it. Therefore, *all kinds of mechanical forces* being only a little too strong have a destroying effect on callus and compression is not an exception to this rule, still less has it a promoting effect on callus formation. Such a stimulating, favourable effect is only apparent

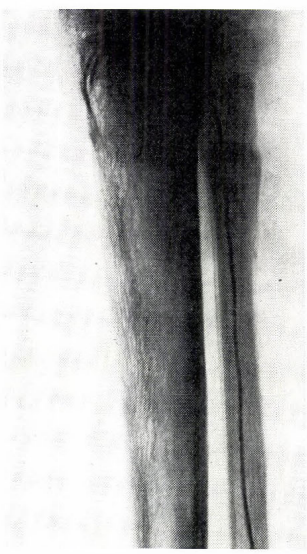


FIG. 2. New formation of fine periosteal bone layer owing to chemical inflammation induced by rusty wire (human fibula)

as the fragments compressed to each other are better protected against lateral displacements, so that by compression a greater mechanical rest ensues in the fracture gap. It is just the absolute immobilization that is needed by the young callus tissue for its development, i.e. protection from greater deformations which would cause its tearing apart. In the experiments with rusty wire or sulphur, demonstrated in the foregoing, the bone tube was intact. Here the development of the callus was not disturbed by mechanical influences.

Thus, a *mechanical differentiation,* in the sense of Roux's theory, that compression forces bring about bone formation, while traction forces evoke formation of connective tissue *does not exist.* This is quite impossible because there is always a bi-axial tension during mechanical employment of bone, e.g. where the primary tension appears as compression force there is always a secondary traction force demonstrable at right angle to it and vice versa, where the primary force is traction there always exists a secondary compression force at right angle to it. This statement is in agreement with the fine experimental results of Matzen on the effect of compression. No *hydrostatical compression* can be demonstrated here; in this respect I am entirely in agreement with Krompecher.

The difficult problem of differentiation may thus be replaced by the simple principle of maintenance or destruction.

The practical conclusions drawn from these recognitions are of utmost importance. Bony healing can be achieved only if prolonged and uninterrupted immobilization is ensured. This has long been recognized in clinical practice and it is known as *Böhler's fundamental principle.* The best way of immobilization of a fracture gap can be achieved with a properly applied medullary nail. The nail should be driven elastically into the broken fragments, and lengthenings which occur even in strongest mechanical strain in the fracture gap are considerably slighter than the deformations manifested in the unbroken bone owing to identical strain. Even if the fracture gap opens about 18 mm, bony healing will eventually occur.

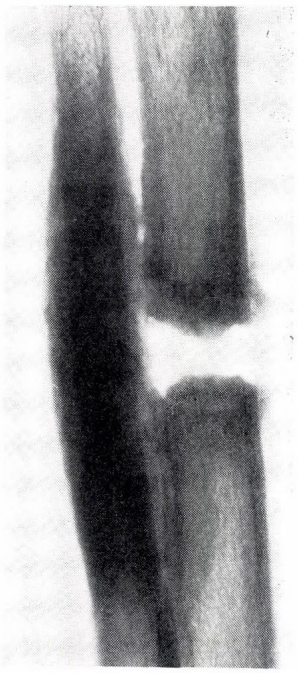


FIG. 3. Martin's experiment

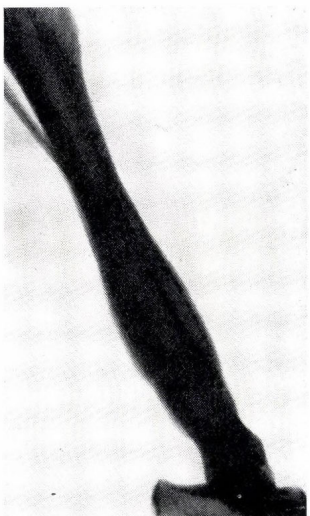


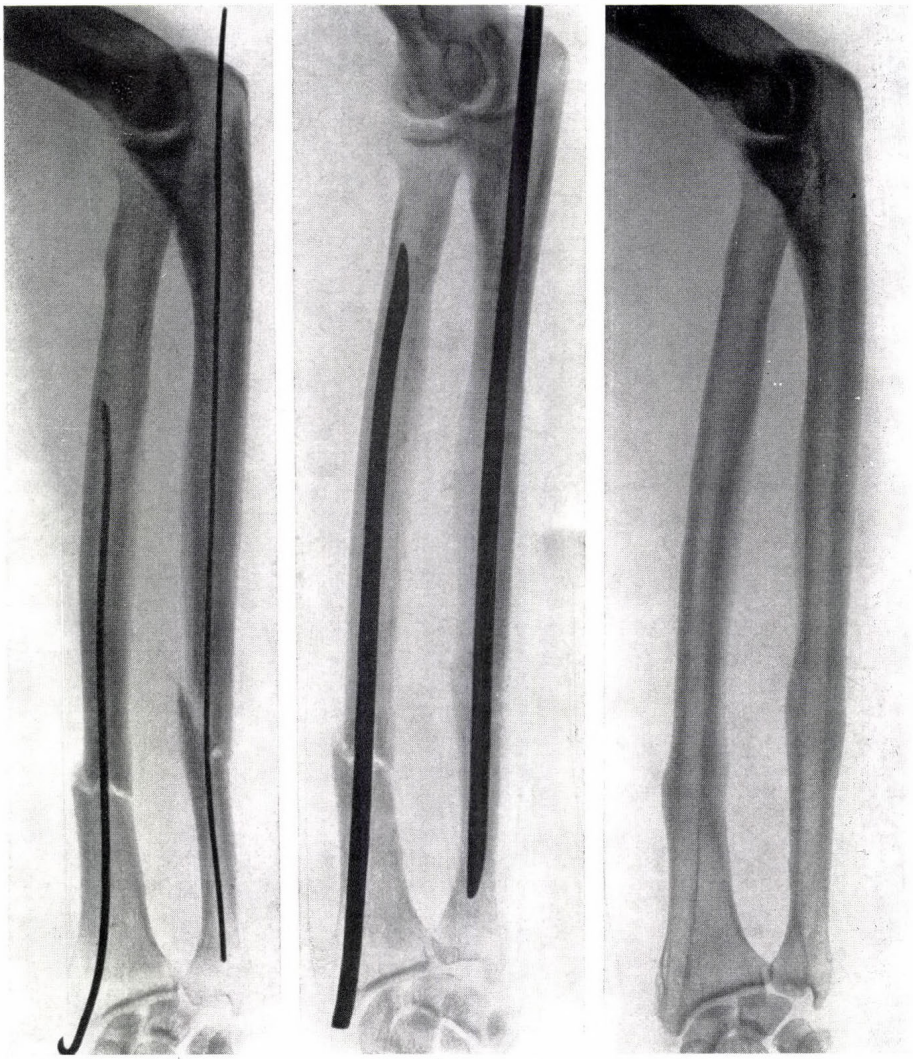
FIG. 4. Callus formation in chronic inflammation

The fracture which was practically perfectly immobilized shows a very interesting histological pattern. We have been accustomed in fracture treatment with plaster or distraction bandage that the small osseous spicules show an irregular arrangement in the fracture gap. Here we also find cartilaginous and membranous islets, the first signs of the provisional callus. In complete immobilization by medullary nailing as well as in calluses developed following chemical stimuli, no such preformative stages are found since bony calluses are obtained here directly.

The Swiss scientific working team on osteosynthesis *(Arbeitsgemeinschaft für Osteosynthese)* has developed, during very carefully conducted experiments, a method consisting of screwing together the broken fragments, by which absolute immobilization was likewise achieved. Here, too, no preformative callus has developed. This process of fracture healing has been termed by this working team 'primary bone formation'. The term is appropriate, but it may be misinterpreted as primary wound healing and, therefore, the term 'direct bone healing' seems to be more suitable. As Griessman and Reich demonstrated histologically in 1941, the preformative stages of cartilage and connective tissue are lacking in fracture healing following medullary nailing. The arrangement of the osteons showing a strict parallelism is very impressive. The same parallel pattern is obtained with medullary nailing, following chemical stimuli and in Martin's experiment. The osteons are arranged along the blood vessels which have arisen as a result of inflammation. According to Krompecher, we have to deal here with angioplastic calluses (as has been formerly mentioned, function has no effect here; it is the inflammation which is responsible for hyperaemia and increase of vascularization). No such features have been noted by the Swiss researchers who found only very slight amounts of periosteal callus or none at all in direct bone healing, and they consider this finding as a characteristic feature of this type of fracture repair. However, an objection may be raised here, namely that by applying numerous bone screws in the course of operative procedure and by the large wound produced, the conditions for healing may be considerably disturbed and the periosteum may be unable to develop such a callus.

These theoretical considerations have been splendidly confirmed by clinical practice employing closed medullary nailing. In more than 800 cases immobilization of the fracture gap by proper medullary nailing proved to be sufficient for repair. Freshening of the fracture ends, clearing of the pseudarthrosis gap, grafting of bone splinters, plaster cast—formerly in use—all proved to be superfluous, even having a damaging effect. As an example I wish to present a case of pseudarthrosis of the forearm arisen following medullary nailing. Although the thin wires have prevented the lateral shifting of the fragments, satisfactory immobilization of the fracture gap was not ensured. By a simple replacement of the thin wires with proper medullary nails and by widening the medullary cavity good repair was achieved (Fig. 5).

No doubt, *undisturbed function* is of considerable importance in bone healing in the sense that blood circulation remains untouched and is even stimulated by muscular action. Finally, the healing processes may be greatly



a b c

FIG. 5. Pseudarthrosis of the fore-arm formed after medullary nailing, treated by widening of the medullary cavity and by closed nailing; a = prior to intervention; b = soon after it; c = 2 years later, immediately after removal of the nail

affected by a local circumstance, namely, whether or not the wound has been opened. Non-opening of the fracture site is very important in the treatment of fractures. Even 30 years ago Götze and Brakertz pointed out that all operative reductions are associated with an increase of local acidity

owing to handling of soft parts, by which the danger of infection is augmented and healing is retarded. This can be avoided if closed medullary nailing is performed or internal osteotomy, i.e. if osteotomy is carried out through the medullary cavity. In this way the large open wound with all dangers of postoperative infection, especially the increase of local acidity, can be avoided. Amazing results have been obtained: extraordinarily rapid healing with unusually accelerated filling of defects, resorption of bone protuberances and rapid reconstruction.

Symp. Biol. Hung. 7, pp. 159–163 (1967)

FUNCTIONAL CALLUS FORMATION IN ANKLE FRACTURES

by

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AN OPEN operation is frequently performed on severe comminuted closed fractures of the ankle, since in case of failure of closed reposition early arthrodesis of the ankle is necessary. The result is usually satisfactory, because the movement of the talocrural joint—according to the biomechanical rule of equilibration of movements—is well replaced by the neighbouring tarsal and metatarsal joints, and after a time the rigidity of the ankle is hardy noticeable.

Undoubtedly, this operation has its disadvantages, too. It is not so much the surrounding scarring or the circulatory disturbances (swelling) that cause difficulties, but the fibrous adhesions developing sometimes at the site of operation owing to necrosis of the talus or to the wrong operative technique. The result is a painful, complete or half arthrodesis.

CASE REPORT

We wish to report on a case not only because the patient after dramatic antecedents showed a striking healing, but first of all, because she was treated by directed active movement therapy, instead of the conventional operative and plastering method.

B. G. a 53-year-old woman tried to commit suicide by throwing herself from the upper story of the hospital where she was treated for coxitis tuberculosa by performing an ischiofemoral arthrodesis on the right side. After the fatal leap the patient was in such a desperate condition that in the absence of the chief surgeon she was transported to our Clinic.

The patient suffered the following injuries: comminuted fracture of the left humerus at the level of the surgical neck; an oblique fracture with dislocation at the site of the ischiofemoral arthrodesis; complete transverse fracture in the proximal third of the right leg; comminuted central fractures in both ankles.

TREATMENT

Reposition of the fracture of the humerus and placement of the arm on an abduction splint. Initiation of directed active movement therapy.

The right lower extremity was immobilized by a hip spica for a period of eight weeks, considering the site of the arthrodesis and the fracture of the

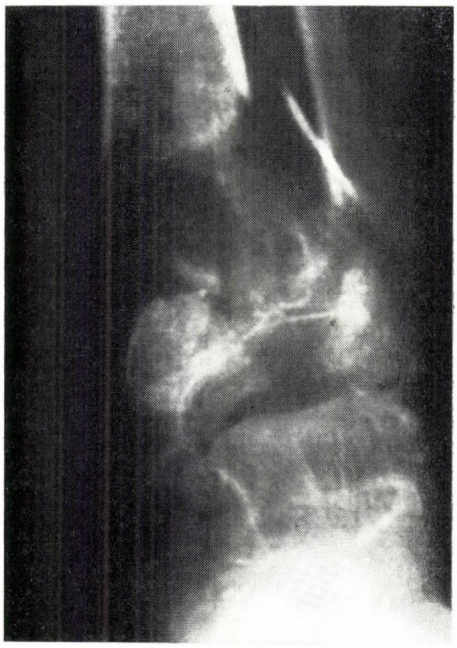

FIG. 1. Anteroposterior roentgenogram of the less severely fractured (right) ankle

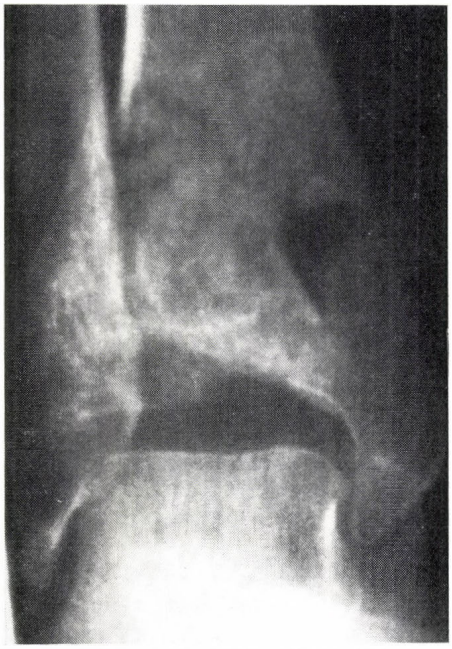

FIG. 2. Severe fracture of the left ankle

upper third of the leg. It should be mentioned that the fracture of the ankle on this side was relatively less severe than on the left side (Fig. 1).

In the case of the left fractured ankle, we intended to perform an arthrodesis in the talocrural joint after a couple of weeks and until that time we thought to treat the injury by our new method mentioned above (Fig. 2). The reposition of the fracture was performed by extension with a screw and passing a Kirschner wire through the calcaneum. After reposition, directed active movement treatment was started.

The effect of the treatment of extension and movement was striking, the swelling of the ankle subsided and the patient was gradually able to move her ankle without pain. Further treatment consisted of the application of a U shaped plaster cuff (in a transverse direction) around the ankle. The roentgenograms taken 8 to 10 days later revealed a rather close re-adjustment of the fragments (Figs 3 and 4), probably due to motion. In the third week, after removal of the extension and plaster cuff, a wooden box was placed in the bed against which the patient pressed her leg with increasing force. In the ninth week the patient was set on her feet and made to walk (Fig. 5).

DISCUSSION

For about six years we have performed in our Clinic, on the initiative of Professor Pap, the directed active movement therapy in fractures of

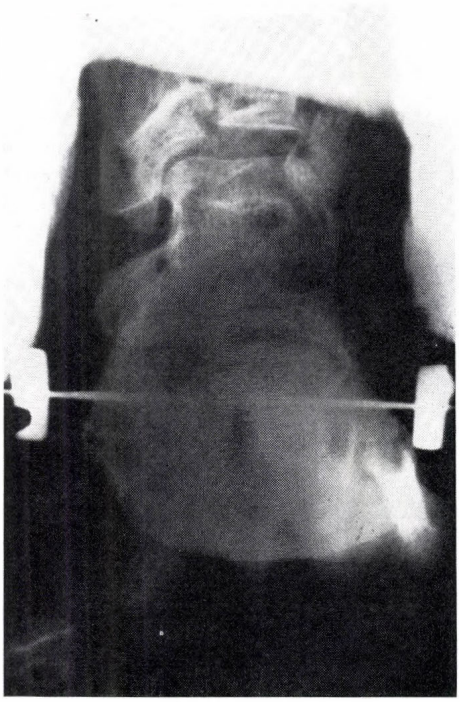

FIG. 3. Anteroposterior roentgenogram of the left ankle with a plaster cuff, treated by active movement therapy. The fractured fragments are almost re-adjusted.

various bones (clavicle, upper third of the arm, scapula, collum scapulae, pelvis, femur, patella, knee-region, lower third of the tibia) and obtained good results.

The introduction of this treatment was stimulated by the observation of Andreesen who stated that fractured fragments often re-adjust themselves owing to the effect of extension and movement.

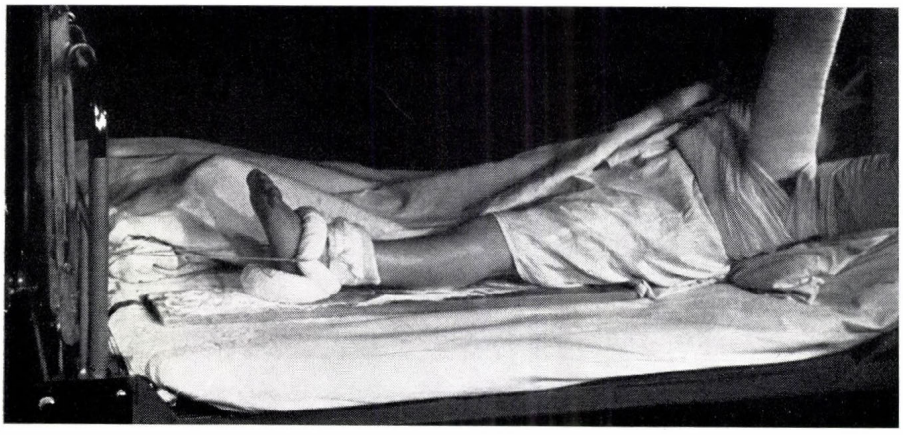

FIG. 4. Exercise with extension

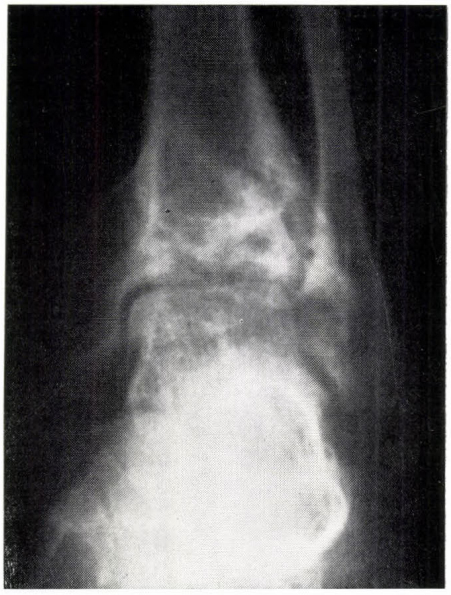

FIG. 6. Roentgenogram of the talocrural joint one year following the accident

FIG. 5. Weight bearing of the patient is painless

FIG. 7. Movement of the left leg

It is known that the fibrous tissue filling up the small unevennesses of the articular surface transforms to fibrous cartilage under the influence of motion. Therefore, in a number of cases accurate reposition is not essential (Landells). After these considerations it seemed reasonable to start the treatment, knowing that in case of failure, the usual talotibial arthrodesis could be performed at any time. One year later, at the control examination, the patient walked without pain and said that she was able to do her housekeeping undisturbedly (Figs 6 and 7).

SUMMARY

One of the bilateral comminuted ankle fractures was treated with directed active movement therapy. The functional result of the moved (left) ankle was better than that of the unmoved (right) one.

At present we have reported, only on one case considering it to be of some interest, but the experience gained in our Clinic with more than 300 fractures may give a stimulus to those who are interested not only in the mechanical factors, but also in the biomechanical aspect of fractures and wish to contribute to the further development of the treatment of fractures.

Symp. Biol. Hung., 7, pp. 165–177 (1967)

TOPOGRAPHIC AND TEMPORAL CORRELATION OF PROCESSES OF OSTEOGENESIS DISCUSSED ACCORDING TO ELECTRON-MICROSCOPIC FINDINGS*

by

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IN A report concerning electron-microscopic investigations of osteogenesis, we must keep in mind that the possibilities of each method are limited. Therefore, we had to compare in this work findings obtained with various methods.

Osteogenesis takes place in a small stratum of less than 1 μ in diameter. The resolving power of the electron microscope of 20 to 40 Å, compared with that of the light microscope of 5000 Å, permits, to some extent, the determination of the topographic localization of the synthesis of the various components of bone tissue.

There are relatively few electron-microscopic investigations on osteogenesis, above all, extensive investigations on the callus are scarce. Moreover, due to the rapid development of the electron-microscopic technique, numerous works have now become insufficient technically.

First we have to discuss what we mean by osteogenesis. According to our present cytological and biochemical knowledge, osteogenesis is described as co-operating processes which synthesize all the components of bone tissue. This statement is simple, but it is difficult to say which substances belong to the bone tissue. There are substances which are characteristic of bone and others which, deriving from the blood plasma, have migrated in the bone and other connective tissues. We must distinguish between the cells and their proteins, polysaccharides, fat, etc., and the intercellular substances. In addition, there are fibres, mucopolysaccharides, heteropolysaccharides, proteins, soluble collagen, minerals, water, as well as proteins and enzymes from the blood (Eastoe 1956, Gersh and Catchpole 1960, Neuman and Neuman 1958). Many of these tissue components appear first as intercellular substance, prior to osteogenesis, as has been definitely confirmed by electron-microscopic investigations.

The first electron-microscopic investigations (chicken: Jackson in 1954; enchondral: Scott and Pease in 1956; Robinson and Cameron in 1956; periosteal: Knese and Knoop in 1958; intramembraneous: Ascenzi and Benedetti in 1959) demonstrated the building of collagen fibrils in immediate contact with the osteoblasts. This is a new formation of fibrils and not a transformation from a pre-existent tissue. Therefore, the term ossification should be avoided and replaced by osteogenesis.

* The original findings concern bovine foetus of 70 to 150 mm CRL.

Numerous authors (Scott and Pease 1956, Knese and Knoop 1958, Ascenzi and Benedetti 1959) pointed out that the diameter of the fibrils is small and the period is less than 640 Å. Today this statement is of no great importance since fibrillogenesis is investigated exclusively by the reconstitution of fibrils (Knese and Knoop 1958). Knese and Harnack (1962) and Knese (1963b, c) investigated the growth of fibrils regarding their diameter and period. A distinction should be made between crystallization and maturation of fibrils; the latter process is still rather obscure.

There is no doubt today that the synthesis of the chemical building materials of the future intercellular substances is bound to the cell. Years ago the release of the intercellular substances from the mature osteoblast has been described as a kind of secretion. Knese and Knoop (1961c) pointed out that as regards cell organelles, gland cells and osteoblasts are similar, being cells that synthesize and secrete substances. There is, however, a fundamental difference between these cells, because in the former case the secreted material leaves the gland, but the substances released from the osteoblast remain in the tissue and participate to a large extent in histogenesis.

The endoplasmic reticulum of the osteoblast has the size of that of an exocrine pancreas cell. Therefore, by electron-microscopic findings the synthesis of scleroproteins can be confirmed in the osteoblast (Figs 1 to 3).

There has been much debate concerning the synthesis of mucopolysaccharides in the periosteum and the role of these substances in fibrogenesis and mineralization, but no clarification has been attained. The deposition of ^{35}S and metachromatic reaction in the periosteum is known. The metachromasia of the periosteum is, however, described differently by the authors.

The question arises whether a single cell, the osteoblast, is able to synthesize these complex substances at the same time. Therefore, this should be investigated with the cell organelles as equivalents for the synthesis mentioned.

The osteoblasts develop in the cambium layer from the *precursor* cells. The cells which are in a transitional stage are called *pre-osteoblasts* (Pritchard 1952). We suggest to distinguish, besides precursor cells and pre-osteoblasts another type of cell, the *cambium* cells (Knese).

The size of the precursor cell is like that of the fibroblast. On the demarcation line between the fibro-elastic and cambium layer, there are cells among large amounts of collagen fibres, having nuclei on one side of their bodies. By light microscopy it is often very difficult to distinguish between the cell body and the intercellular substances. These cells have a gradually developing endoplasmic reticulum, isolated ribosomes, mitochondria and Golgi-complexes. Other cells, the precursor cells, enclosed in a loose framework of fibrils, contain groups of granules contrasted with lead. These granules may be polysaccharides. By light microscopy, an increase in storage of stainable polysaccharides from the precursor cells to the pre-osteoblasts could be demonstrated.

The cambium layer at some distance from the fibro-elastica contains only a few collagen fibrils or none at all. On the other hand, it contains large intercellular spaces. In these spaces, long digitate cell processes are visible in cross-sections or longitudinal sections. The cells are considerably ramified,

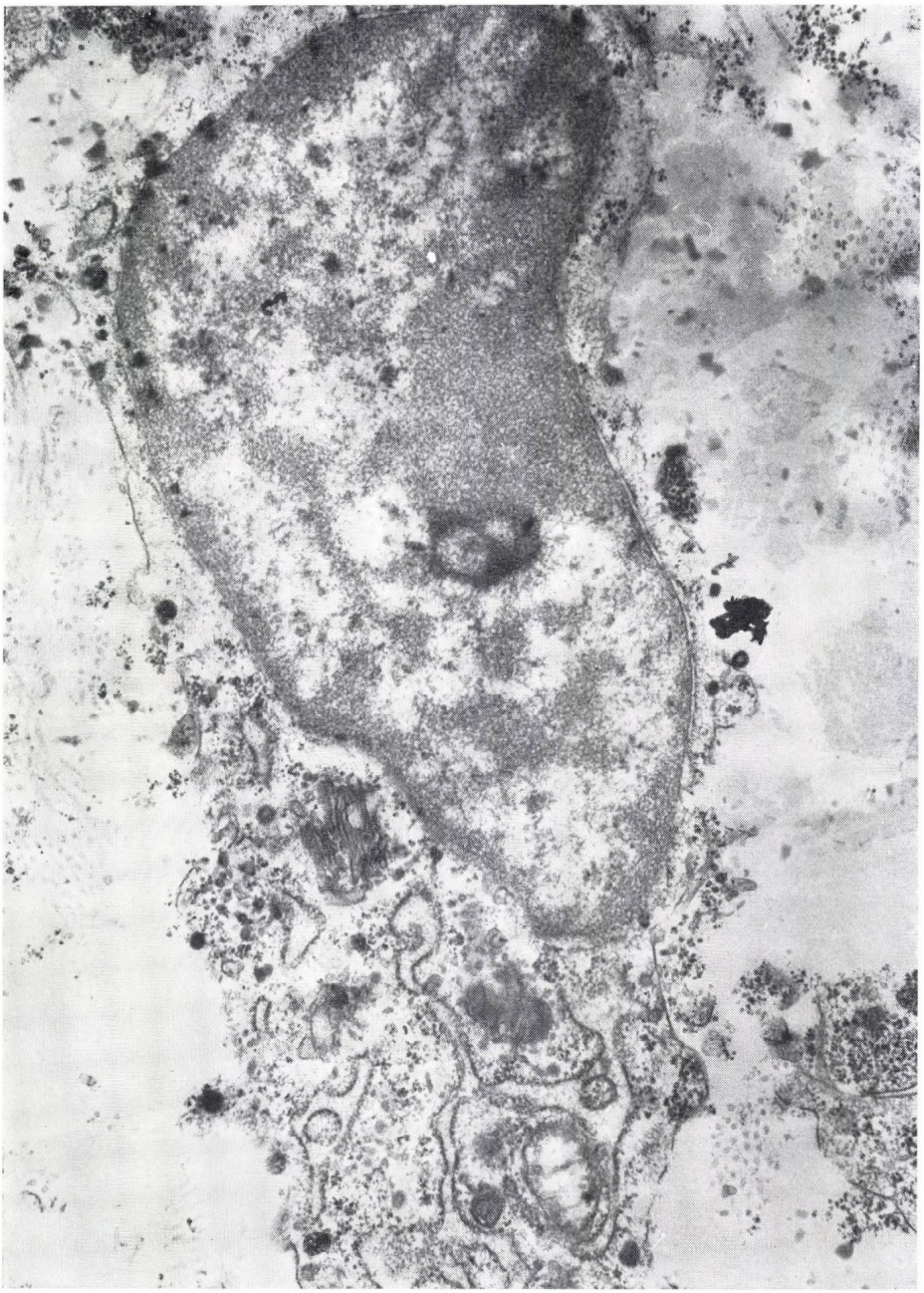

FIG. 1. Fibroblast-like cell with slowly developing endoplasmic reticulum, ribosomes, mitochondria, Golgi-complex, few lead-contrasted granules and nucleolus; ×10,000

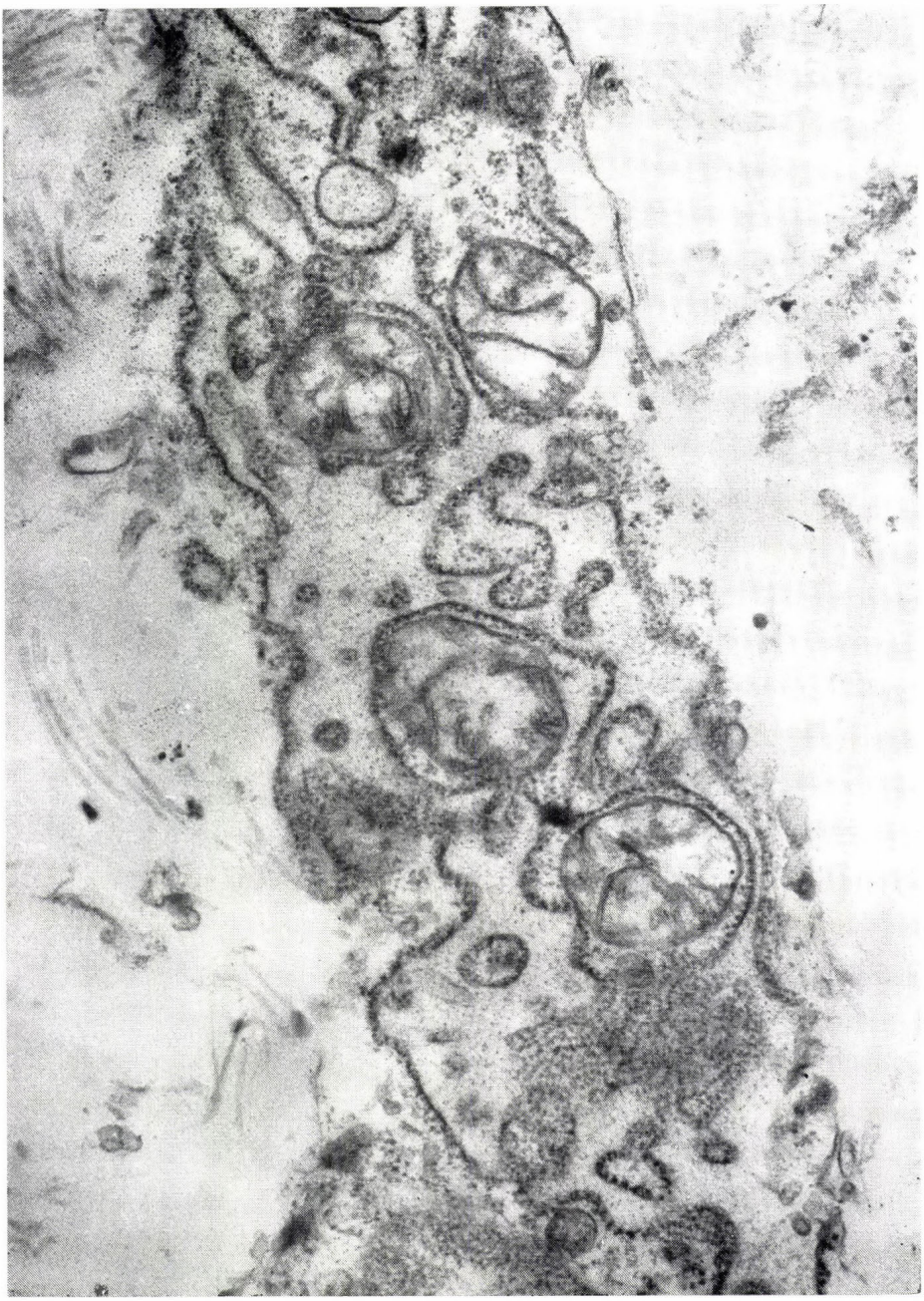

FIG. 2. Precursor cell with enlarged cisternae of endoplasmic reticulum and mitochondria; ×19,000

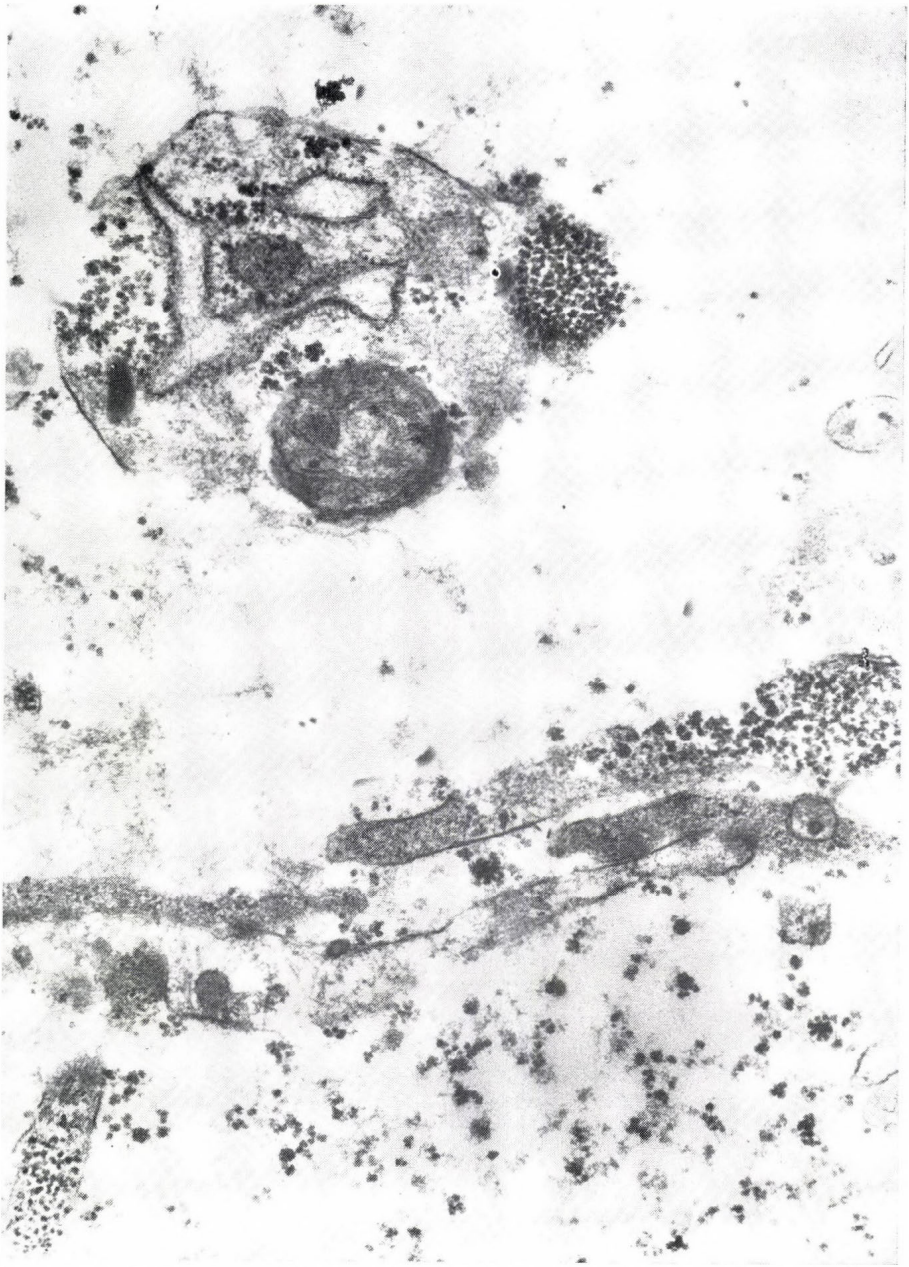

FIG. 3. Cambium cells. Smaller and larger digitated processes with endoplasmic reticulum, mitochondria and lead-contrasted granules. In the large intercellular spaces filaments and lead-contrasted granules; × 18,000

so that no cell border can be verified with the light microscope. The intercellular spaces contain a few thin filaments and groups of granules contrastable with lead. In the cells, the cisterns of the endoplasmic reticulum are enlarged, and the hyaloplasma with the mitochondria is compressed to small clefts. The enlarged cisterns are connected with the perinuclear cistern. The nucleus contains large amounts of chromatin in contact with the inner nuclear lining, the central area of the nucleus being nearly empty. These cells exhibit numerous nuclear pores of considerable width. Such cells are termed by us 'cambium cells' (Fig. 4).

These cambium cells with enlarged cisterns are very similar to chondroblasts. This structure of cells has been regarded by us (Knese and Knoop 1961a) as equivalent to mucopolysaccharide-protein synthesis. We have demonstrated (Knese) intracellular and extracellular metachromatic granules after fixation with alcohol or formaldehyde, deparaffination in vacuum and staining with thionine, azure A and B. The same staining was displayed by the chondroblasts.

These electron microscopical and topochemical findings lead us to the presumption that the synthesis of mucopolysaccharides takes place in the cambium cells, but probably no more in the pre-osteoblasts. As acid and partly neutral polysaccharides are secreted in the intercellular spaces, we have regarded the thin filaments seen in these spaces by electron microscopy as mucopolysaccharide-protein structures. The cambium cells are not only 'differentiating' cells developing the structure of the future osteoblasts, but also cells which produce intercellular substance. The pre-osteoblasts near the mature osteoblasts appear as longish or ovoid elements under the light microscope. Between the cells, there is a system of small intercellular clefts which are also visible under the electron microscope. These clefts contain a homogenous or small granulated substance with no collagen fibres. These substances react metachromatically. The pre-osteoblasts have shorter or longer digitate processes in large amounts, partly in contact with one another (Fig. 5). Chromatin is approximately regularly distributed in the area of the nucleus, but a compact layer is also in contact with the inner nuclear lining. Small nuclear pores are present. The cytoplasm contains short membranes of endoplasmic reticulum, sometimes a Golgi-complex and regular groups of granules contrasted with lead. The granules may fill out almost the entire cell body.

The mature osteoblast, situated in a pseudoepithelium, has an expanded endoplasmic reticulum, mitochondria and occasionally, a Golgi-complex (Fig. 6.). Only the mature osteoblasts display a clear reaction for ribonucleic acid (RNA) under the light microscope. After treatment with ribonuclease, the mature osteoblast is no longer stainable. The transformation from the pre-osteoblast to the mature osteoblast proceeds without intermediary stages. Morphological observations concerning intermembranous osteogenesis (unpublished) and investigations with tritium-cytidin (Owen 1965, Young 1962) support that the RNA is released from the nucleus to the cytoplasm. By the cytoplasm RNA as messenger of RNA the synthesis of scleroproteins can start. Among the mature osteoblasts, extracellular polysaccharides are missing. By means of topochemical methods, a frame-

FIG. 4. Cambium cells with greatly enlarged cisternae of endoplasmatic reticulum in connection with the cisterna of nuclear lining; small hyaloplasmic spaces with mitochondria; × 15,000

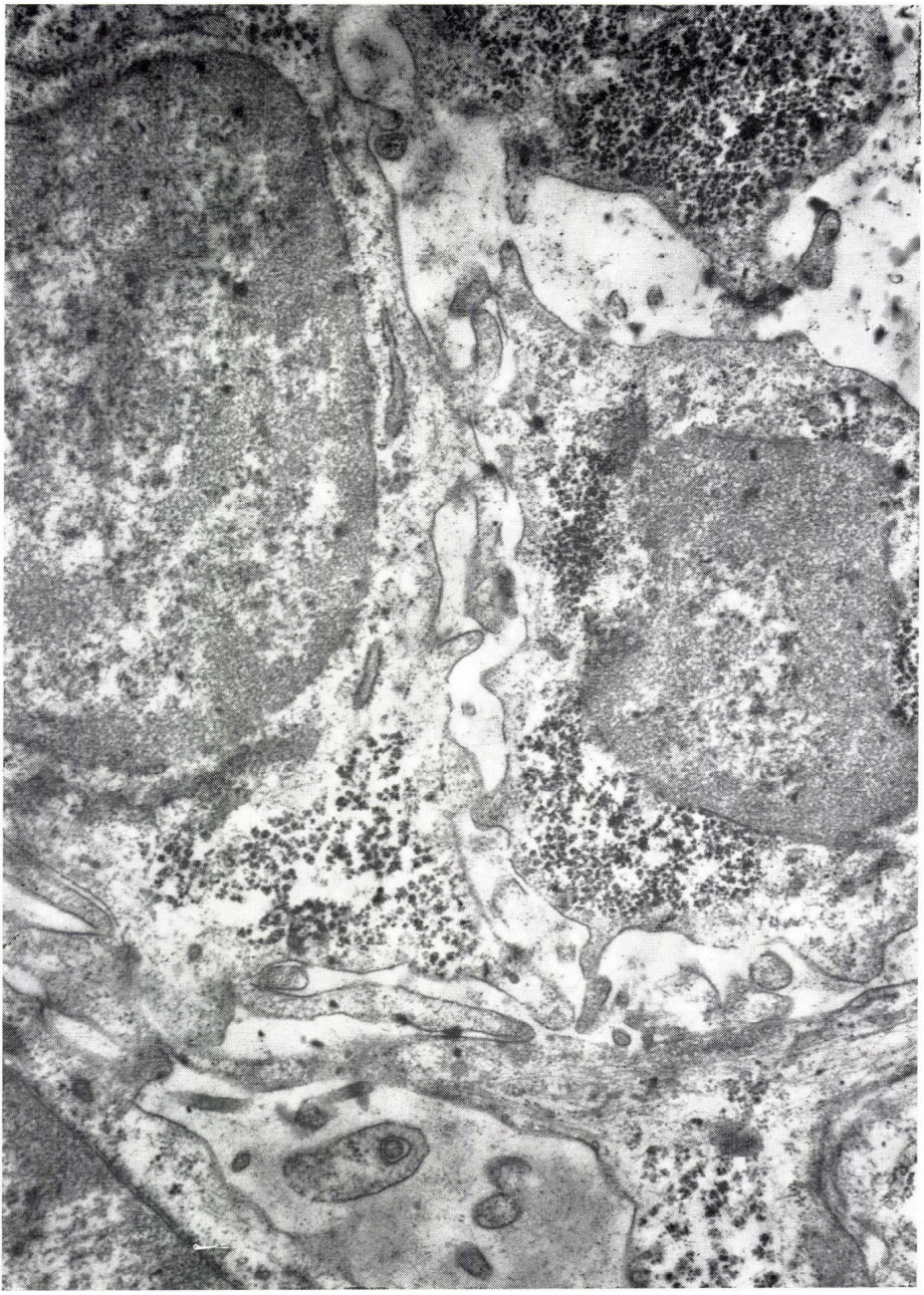

FIG. 5. Pre-osteoblasts with only a few membranes of endoplasmic reticulum, lead-contrasted granules, nucleus pores and many digitated cell processes; small intercellular clefts; × 10,000

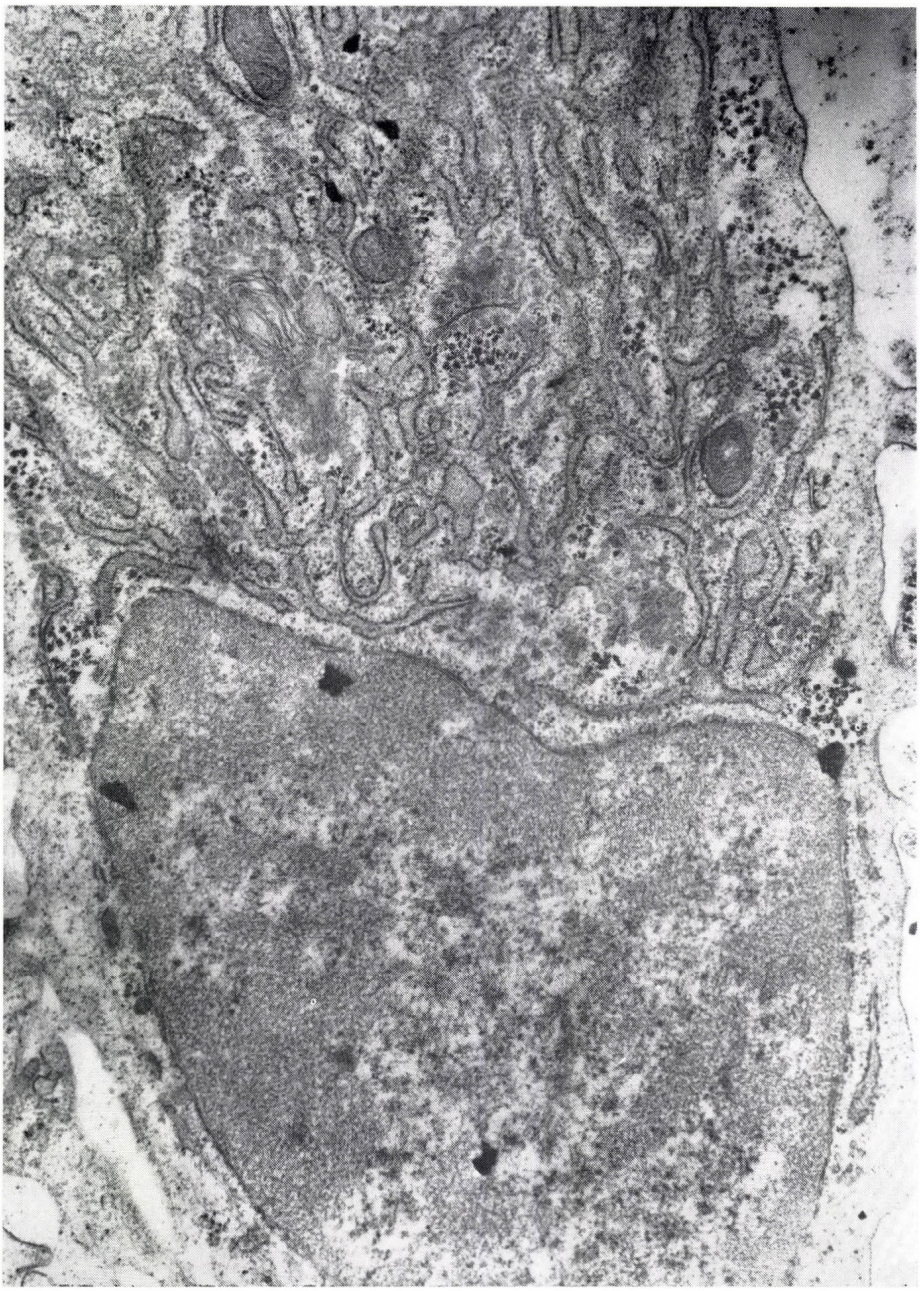

FIG. 6. Mature osteoblast before starting to deliver intercellular substances with an extensive endoplasmic reticulum, a few mitochondria, Golgi-complex, and only a few lead-contrasted granules; × 10,000

work of metachromatically staining meshes can be demonstrated which was considered to be an equivalent of the endoplasmic reticulum (Knese and Knoop 1961b). It is, therefore, supposed that the acid mucopolysaccharides synthesized by the cambium cells are stored in the extracellular spaces. The pre-osteoblast takes up these polysaccharides again by its cell membrane enlarged by large numbers of digitate processes. The mature osteoblast originates from the passage of RNA in the cytoplasm, and absorption of mucopolysaccharides from the extracellular spaces.

From these observations the following conclusions have been drawn:

1. The mucopolysaccharide synthesis does not take place in the mature osteoblast but mainly in the cambium cells.
2. The synthesis of RNA is prepared in the pre-osteoblast with storage of nuclear RNA.
3. The synthesis of RNA occurs in the cytoplasm of the mature osteoblast.
4. The mature osteoblast releases scleroproteins and mucopolysaccharides, probably in a transformed state, before the onset of osteogenesis by which the crystallization of the collagen fibrils will take place.

Thus, the synthesis of these two components of bone tissue, the mucopolysaccharides and scleroproteins, do not occur at the same time and at the same site. Both substances together leave the mature osteoblast and become the intercellular substance of the bone tissue.

Now I wish to return to the comparison of the function of the osteoblast and that of the gland cell. Gland cells have a polar organization and, therefore, an epitheloid shape. The flow of substances occurs from the basis, consequently from the vessels, to the apex which is the site of the release of the secreted material. The phenomenon of secretion is characterized by the transformation of the substances of the blood into the secreted material. Osteoblasts often, but not always, have a polar organization and, consequently, an epitheloid shape. Their activity is temporally limited, probably to three days (Owen 1965), apparently because they do not synthesize a part of those substances which they eliminate.

We must rid our minds of the concept that the osteoblast synthesizes all the components of bone tissue. By applying a temporal and topographical segregation of the processes, a better understanding of the processes of endochondral osteogenesis may be attained, which were up to now incompletely explained.

The metaphyseal osteoblast is similar to the periosteal osteoblast. But —corresponding to its different course of development—the stages from precursor cells to cambium cells and pre-osteoblasts are lacking here. Presumably, the metaphyseal osteoblast absorbs the mucopolysaccharides from the intercellular substance of cartilage and thus, the mucopolysaccharides for enchondral osteogenesis derive from the activity of chondrocytes or even chondroblasts. However, the progenitor cells of the metaphyseal osteoblasts are partly chondrocytes, which—with regard to mucopolysaccharide synthesis—correspond to cambium cells. This fact has been demonstrated by electron-microscopical (Knese and Knoop 1961b) and autoradiographical investigations (Young 1962, 1963).

Some mention should be made of the enclosure of cells as osteocytes. The first electron-microscopical picture of such an enclosure (Knese and Knoop 1958) showed a cell having a few cell organelles, surrounded by a network of collagen fibrils with incipient mineralization. The formulation that the osteoblast encloses itself is, in our opinion, not correct. After the osteoblast has ended its activity, the next generation of osteoblasts surrounds it with bone tissue.

The literature dealing with the osteocyte is extensive and the opinions of the authors concerning the activity of these cells, are greatly divergent, partly dependent on the different methods used by these authors. We have distinguished (Knese 1963a, b, c, 1964) three types of osteocytes: young (just enclosed), polyedric, and flat. The young osteocyte contains only a few cell orgenelles (Knese and Knoop 1958, Baud 1962). The polyedric osteocyte displays an endoplasmic reticulum, mitochondria, eosinophil deposits and a Golgi-complex (Baud 1962). In the vicinity of the polyedric osteocytes Knese and Harnack (1962) observed, partly in contact with the cell membrane, some apparently newly formed, thin collagen fibrils which increased in diameter at a short distance from it. Therefore, our opinion is that the osteocyte participates in osteogenesis, namely in the so-called intra-osseal osteogenesis. This opinion has been supported in various ways by different authors. Baud (1962) who did not investigate demineralized sections believes that the osteocytes alone control the metabolism and the physicochemical condition of the mucopolysaccharides.

The structure of the newly formed bone tissue has been rather poorly investigated. The old scheme of coarse-fibred and fine-fibered, or lamellar bone, has been accepted as a rule in the literature. We have observed not only in our pictures (Knese and Knoop 1961c), but also in those of other authors that the collagen fibrils are arranged in lamellae, not in bundles. These lamellae are similar in their structure to those of mature bone tissue, only smaller in size. Therefore, we have again discussed the formation of the lamellae and the orientated crystallization of collagen fibrils (Knese 1963b, Knese and Harnack 1962). From the topographical distribution of the different types of Haversian systems it was concluded that in normal histogenesis the intercellular substances show a constant development. Amprino (1963) was opposed to this opinion, but almost contemporaneously, Frost (1963) published his findings obtained with tetracycline which confirmed our statement. We are of the opinion (Knese 1963b) that all the investigations of histogenesis of intercellular substances should be substantiated by cytological analysis of the local cell population which is engaged in the metabolism and other processes of the organic components of bone tissue.

Finally, I wish to deal with mineralization. It has always been supposed that mineralization takes place separately from other processes of osteogenesis. This was concluded from the staining properties of the border of the bone trabeculae, the so-called osteoid or preosseal seam. Numerous authors consider mineralization to be the definitive process of osteogenesis. However, it should be borne in mind that the structure of bone is determined by the organic stroma. It is true, that many functions of the bone tissue, mechanical as well as metabolic ones, are only possible if minerals are

present, but in osteogenesis the minerals depend on the organic framework. For many morphological conceptions on mineralization, microradiography of bone tissue was fatal. Owing to the low sensitiveness of microradiography, it is possible to detect the minerals only at a distance of about 20 to 30 μ from the osteoblast. This is the so-called mineralization front.

With the electron microscope, needle-like crystals can be demonstrated at a distance of 1 to 2 μ from the osteoblast. A shell of crystals surrounds all the collagen fibrils. Mineral deposition and the mechanism of mineralization have been extensively dealt with in the literature. The earlier hypothesis of Robinson and Watson (1955) on the primordial deposition of minerals, according the period of collagen fibrils, could not be verified. The minerals have been described as needle-like depositions (Knese and Knoop 1958, Frank and Nalbandian 1963, Ascenzi and Benedetti 1959, Takuma 1963, Glimcher 1959, 1960). A periodical and granulated deposition was described by Jackson and Randall (1956) and by Glimcher (1959, 1960) in chickens. An extensive discussion on mineralization exceeds the subject of the present work.

In the cartilage, the crystals are irregularly deposited mostly forming rosettes (Knese 1959, 1963a). We wish to mention the electron-microscopical investigation on callus formation by Aho and Isomäki (1962) as the only results of this kinda vailable to us. These investigations have been performed on the tibia of white rats. After 10, 14 or 21 days of healing the authors observed a similar rosette built up of needles of a length of 375 to 500 Å and a width of 30 to 60 Å, some needles being only 130×35 Å. These crystals lie in an intercellular substance having an amorphous or granulated structure or displaying thin filaments.

A special form of crystals has been observed by Aho and Isomäki (1962) near collagen fibres of about 0·1 μ thickness and a period of 640 Å. The crystals are located inside the fibres and appear as rather large aggregates attached to their surfaces. They are elongated, spherical or lenticular bodies with diameters of $0{\cdot}1 \times 0{\cdot}3$ μ and partly even above 1 μ. The particles have a compact shell, 30 to 50 Å thick, and a cavity with numerous smaller crystals of 35×130 Å width. It is often difficult to decide whether they are continuous crystals or composed of smaller ones. There are also single crystals along the collagen fibrils. These investigations suggest that in the mineralization of the callus there are processes which differ from normal osteogenesis.

This report is incomplete as regards the subjects mentioned in it and the relevant literature. Its chief purpose was to demonstrate that by means of electron-microscopical findings it is possible to describe the temporal and topographic localization of separate processes of osteogenesis. By this division of osteogenesis in correlated processes, abnormal osteogenesis may perhaps be recognized easier.

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HISTOLOGY AND HISTOCHEMISTRY OF CALLUS IN CASE OF EXPERIMENTAL PSEUDARTHROSIS

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Manuscript not received

Symp. Biol. Hung., 7, pp. 179—189 (1967)

ON THE REGENERATION OF BONE TISSUE EXAMINED WITH TETRACYCLINE IN TRANSPARENT BONE SECTIONS

by

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Tetracyclines form deposits in the bone tissue wherever calcium ions are at disposal in loose chemical bonds. Tetracyclines appear especially in the osteoid seams or in the apposition zones which are not free from calcium as it has been believed up to now, but contain large amounts of calcium ions. They are chelate-like structures related to acid mucopolysaccharides which are present in large quantities in the osteoid seams.

Tetracyclines and calcium form chelates. This combination is, however, not sufficient for the firm fixation of the antibiotics in the bone tissue. Apart from chelate formation, another additional factor is necessary to bring about a durable fixation. Milch et al. (1961) believe that tetracyclines are connected with calcium via an oxygen atom, in a mineralizing tissue (Fig. 1). According to Milch et al. (1961) phosphate is deposited in the collagen fibrils at the beginning of mineralization. This phosphate forms a hardly soluble tertiary phosphate with calcium, and tetracycline is fixed by means of oxygen. The firm and lasting fixation of tetracycline occurs only in the moment of crystallization of the mineral salt. The tetracycline-apatite-complex remains bound to the mature bone tissue and can be analysed even after years. Our observations comprise a period of more than three years. Sedlin and Frost (1962) reported cases in which deposition persisted after nine or eleven years.

The mineralized bone does not take up tetracycline. The tetracycline fixed to calcium in the osteoid, and not involved in the process of mineralization, gradually disappears. In our experimental material when tetracycline was administered in a single dose it disappeared after about 48 hours. This last stage of embedding of tetracycline has been termed by us purified tracing. By this the apposition seam appears to consist of an intensely and permanently fluorescent mineralized zone which is situated at the border of the mature bone tissue. In addition, there is an osteoid zone displaying a less intense fluorescence of a shorter duration which borders the free seam of the bone trabecula or that of the osteon.

The microscopic description and analysis of the fluorescent lines can be made only on non-decalcified bone, since by decalcification the tetracycline is extracted as well. With our method (Eger et al. 1964) transparent bone sections are obtained after embedding of the bone in a plastic material. Any number of transparent bone sections can be cut from the spongious and compact bone with this method. This method enables us to investigate

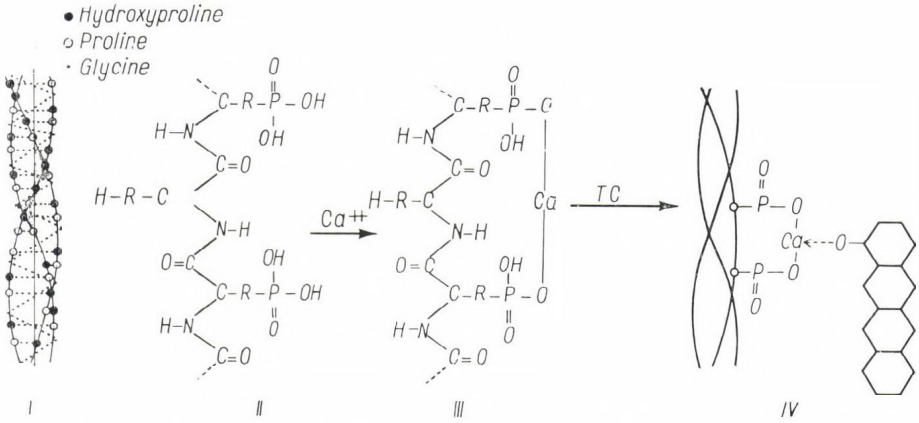


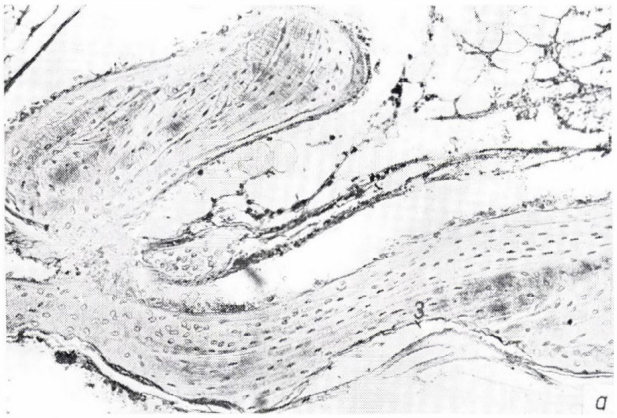
FIG. 1. Binding of tetracycline to apatite crystals and collagen fibres according to Milch. I = a collagenous helix; II = beginning of mineralization marked by the phosphorylation of the polypeptide chain; III = the growth of crystals of apatite by calcium addition; IV = binding of tetracycline to the apatite crystal via oxygen

the growing and remodelling processes of the compact and spongious substance as well. The present opinion is that bone building presupposes decomposition. We agree with Frost (1963) in claiming that *without decomposition no building* exists. The continual turnover of the bone tissue occurs on a molecular and cellular level. The cellular turnover is the basis of bone and calcium metabolism in the organism. In microscopical anatomy the physiological reduction of the compact substance is marked by *resorption cavities*, and that of the spongious bone by *resorption lacunae* (Fig. 2a). This process is brought about not by a giant cell osteoclasia but by mononuclear osteoclastically working cells, termed 'osteolyocytes' by us (Fig. 3) in order to differentiate them from the giant cells. The multinuclear giant cell osteoclast may be seen during an increased local or general decomposition which exceeds the physiological limits.

The resorption lacunae do not expand unlimitedly. Having reached a certain size, apposition zones similar to osteoid seams are again deposited which take up tetracycline. The limiting factor of decomposition is unknown.

If tetracycline is given after longer intervals, all the stages of remodelling can be observed. In the compact substance of a 40-year-old man who received tetracycline twice within six months, a resorption cavity was found showing the greater part of the slightly lacunated margin of the decomposition zone, and a part of a fluorescent apposition zone as well. Other resorption cavities were completely surrounded by apposition zones. In addition, there were completely built osteons, the outer contours of which consisted of an old cementing line (Fig. 4).

Under physiological conditions about 1 μosteoid is formed daily. Mineralization of the whole lamella starts suddenly, after a period of ten days, preceded by a loss of acid mucopolysaccharides (Eger 1963a, b). The lamellae are



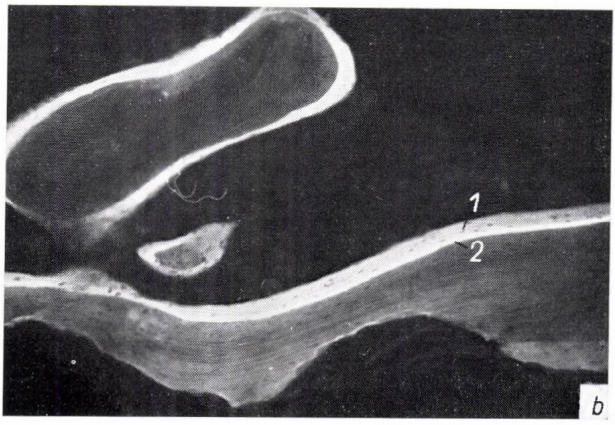


FIG. 2. Transparent section of spongious bone (male, aged 34); a = in transmitted light; b = in ultraviolet light. The osteoid seam is hardly visible (a), whereas a wide fluorescent margin with a more intensive mineralization zone is seen on the border of the maturing bone tissue (b). 1 = maturing bone showing fluorescence; 2 = intensive mineralization zone (stronger fluorescence); 3 = resorption lacuna

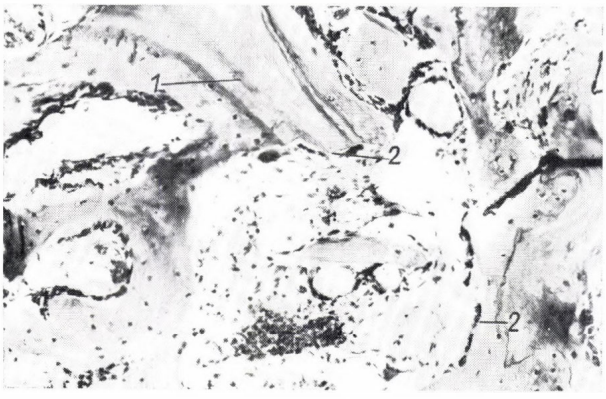


FIG. 3. Resorption and regeneration around a homologous transplant. 1 = necrotic parts of the transplant; 2 = resorption of the transplant by single giant cells and by osteolyocytes developing a slightly undulated resorption margin

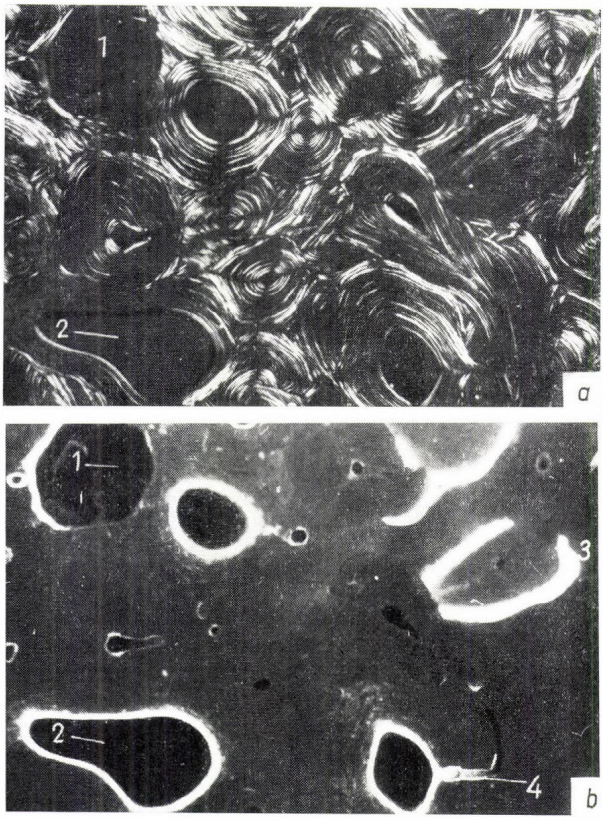


FIG. 4. Compact bone (male, aged 40) and fresh trace of tetracycline just before resection of the bone tissue; a = in transmitted light; b = in ultraviolet light. 1 = resorption cavity partly surrounded by a traced margin of 55 μ dia; 2 = completely surrounded resorption cavity, beginning of growth of the osteon; 3 = completely built osteon with fluorescent outer contours of 30 μ dia; 4 = traced margin of a Volkmann's canal

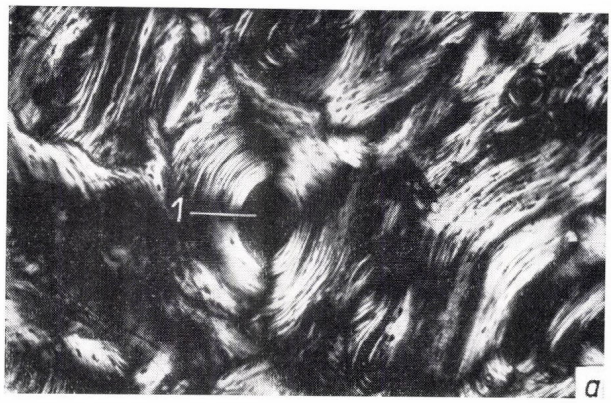
arranged side by side. Within 8 to 12 weeks, a human osteon comes into existence. Such an osteon has only one Haversian canal in it with a lumen of 20 μ.

In our material we have observed in a 35-year-old man osteon formation lasting three years at the site of a bone fracture (Fig. 5). The outer trace line of about 40 μ dia came into existence three years ago in the course of four months in which tetracycline was given to the patient. The interior line (25 μ dia) was produced about 18 months ago due to tetracycline administration for two months.

Manson and Waters (1963) expressed the growth of the osteons by a mathematical formula and gave a diagrammatic illustration of it. On calculating the growth of the osteon described above, by means of this formula, a diagram is obtained (Fig. 6) which shows that growth slows down in the centre and finally stops. There seem to be remarkable temporal differences in the formation of the osteons, i.e. considerable retardations of growth in the zones of regeneration.

On the side of the trabeculae of the spongious substance more apposition zones have been demonstrated by the tetracycline method than was supposed until now. The number and breadth of the traced zones do not only indicate the speed of growth but also the rate of the local metabolism, especially that of mineralization of the bone tissue.

As has been emphasized above, the increased decomposition of the bone tissue is preferably done by osteoclasia of giant cells with Howship's lacu-



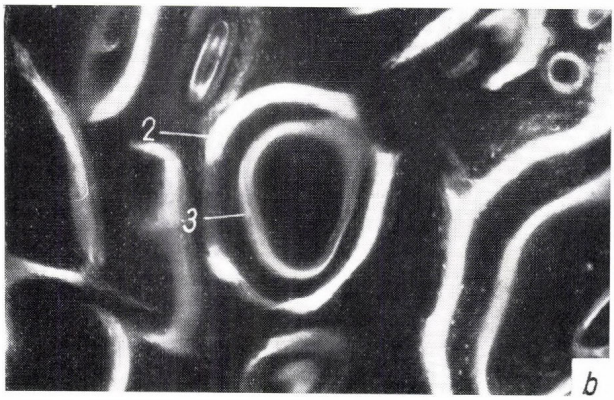


FIG. 5. Compact bone (male, aged 32); a = in polarized light; b = in ultraviolet light. 1 = in the centre a completely built osteon (400 × 320 μ); 2 = at the outer border a fluorescent zone (40 μ wide) which was formed 3 years prior to the resection; 3 = a trace zone of 15 μ formed 18 months before the resection

nae. Neighbouring osteoclasts in the zone of disturbance form a confluent layer on the surface of the bone. This layer appears in cross-section as a rather narrow lacunated line on the border of the bone. Here calcium ions are released which are able to fix tetracycline. This tracing is temporary during decomposition. The example, however, shows that not every fluorescent seam represents a zone of growth, but it may likewise appear during decomposition processes of living bone. The morphological differentiation between a zone of building and that of decomposition is easy to perform (Fig. 7).

Halisteretic demineralization, as the first phase of another form of decomposition of bone, is caused and maintained by a local and general acidosis. By this demineralization large amounts of calcium ions are released which are able to combine with tetracycline. Under these circumstances a diffuse fluorescence appears spreading over the whole tissue. From our material we mention a compact Kiel type splinter of bone graft which had been implanted to fill up the defect formed after the surgical removal of a cyst 18 months ago. This graft showed a diffuse, partly spot-like fluorescence following tetracycline administration (on three occasions), prior to its resection and examination (Fig. 8). We do not know at present to what extent halisteretic demineralization takes place in living bone tissue. No observations referring to this point have been made in our experimental material either. Presumably, halisteresis in living bone tissue leads to an

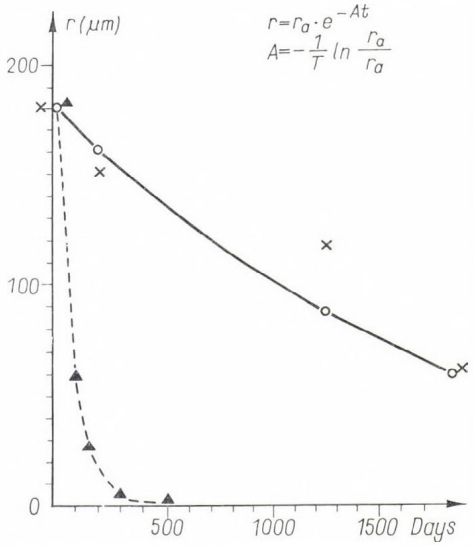


FIG. 6. Course and speed of growth of the osteon, built in 3 years, compared with the average time of growth of an osteon observed by Frost (dotted line). o = calculated results; x = measured results

immediate removal of calcium ions. This is why no impregnation of tetracycline and, consequently, no fluorescence are visible in the histological preparations.

When the bone autograft becomes necrotic as demonstrated in examinations (Fuchs et al. 1963), and when at least some of the splinters of bone within the region of bone fracture become necrotic, it is supposed that the necrotic autograft will also be demineralized by halisteresis and, in this way, a temporary uptake of tetracycline may result.

The final decomposition of the heterologous and autologous necrotic bone shows distinct histological differences as demonstrated by the spongiosa test of Maatz et al. (1954) in our investigations (Fuchs et al. 1963). Vivid osteoblastic and osteoclastic activities start around the homologous necrotic bone. Osteolyocytes as well as giant cell osteoclasts are present, i.e. all the characteristic elements of increased physiological decomposition.

As has been mentioned above, the heterologous graft shows only halisteretic demineralization and no lacunar giant cellular resorption. Therefore, the decomposition of this bone graft cannot occur in a physiological way, but takes place rather by means of demineralization and a gradual dissolution of the ground substance, i.e. by histolysis. The entire process develops slowly. The physiological decomposition of a bone autograft occurs more quickly by lacunar resorption and is rapidly substituted by living bone tissue.

In both cases the necrotic bone is replaced by a regenerating tissue from which fibrous bone is first developed. While the autograft bone shows a continuous substitution, the compact and spongious heterologous bone graft is surrounded by fibrous bone which forms at all those sites where an increased remodelling process of bone tissue takes place (Fig. 9).

In polarized light the fibrous bone shows a coarse bundle diffusely impregnated by tetracycline. This diffuse impregnation is probably due to the fact that the apatite crystals are not closely and densely packed in the fibrous bone as they are in the mature lamellar bone tissue. Consequently, the fibrous bone is permeable for tetracycline throughout. There are numerous hydrated calcium ions which are loosely deposited on the surface which may combine with tetracycline.

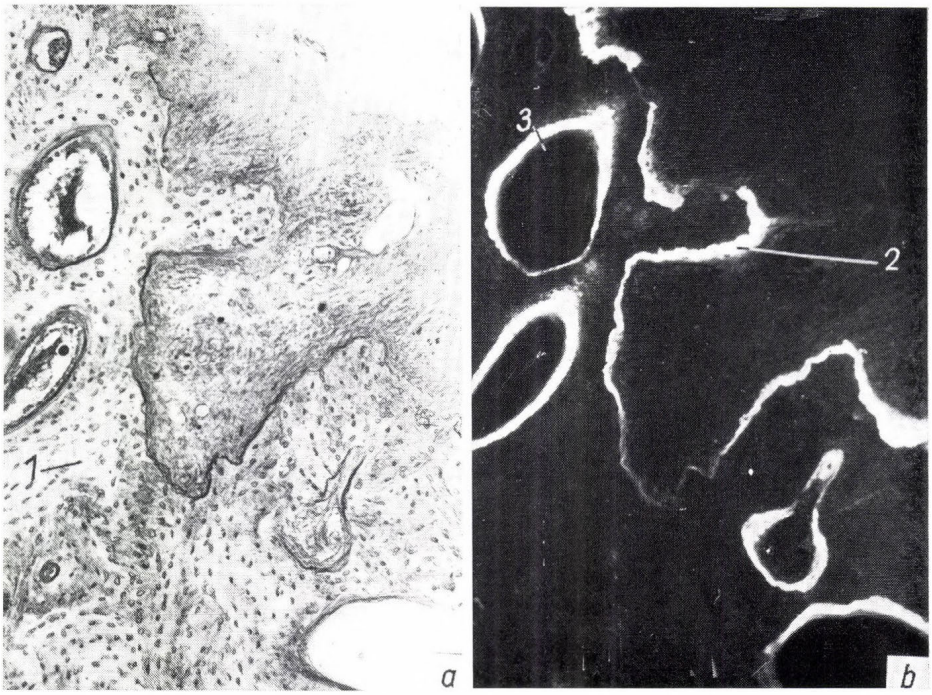


FIG. 7. Transparent section of the compact substance of the thigh bone; a = in transmitted light; b = in ultraviolet light; 1 = compact substance of the ossifying bone fibroma with processes of reduction at the margin; 2 = traced line of resorption; 3 = traced apposition zones in resorption cavities

Fibrous bone continuously transforms into lamellar bone tissue. The lamellae form short bundles of fibres which are linked together and show a mosaic-like structure as seen in polarized light. This structure is not identical with that described by Paget. Fibrous bone is not decomposed in order to be replaced by lamellar bone tissue, but to be transformed into it.

We have observed in the healing leg of a 62-year-old man, whose leg was fractured three months prior to the resection of a small piece of bone tissue, a fluorescence due to tetracycline administration soon after the fracture (Fig. 10). The impregnated callus tissue appeared in polarized light to consist of fibrous bone, whereas the bone tissue in the vicinity of it and which has developed later, showed a short lamellar structure, i.e. the structure of maturing regenerating bone. The described rebuilding of short lamellar regenerating bone is inhibited by tetracycline impregnation.

The autograft which becomes necrotic and is destroyed and replaced by newly built bone tissue, still maintains the structure of the regenerating bone for a long time. An autologous fibula graft, transplanted into a resected radius two years previously and completely ingrown there, showed a short lamellar and mosaic-like structure in polarized light (Fig. 11). While we may

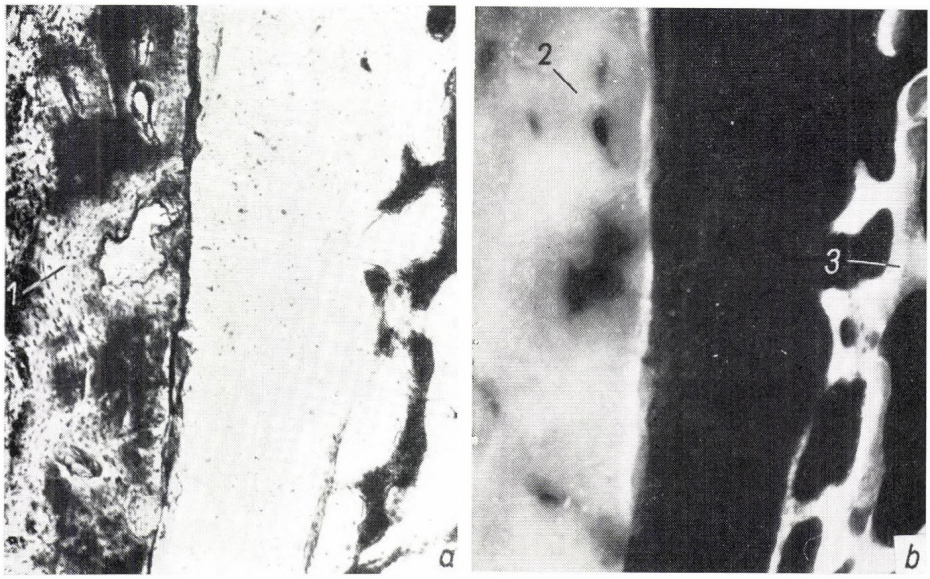


FIG. 8. Compact, heterologous transplant (Kiel type bone graft); a = in transmitted light; b = in ultraviolet light. Traced with tetracycline 3 days before the resection. 1 = the dead compact graft; 2 = showing a diffuse or spot-like fluorescence in u.v. light; 3 = diffusely traced regenerative fibrous bone of the graft bed, separated from the Kiel type graft by a wide zone of connective tissue

find trace zones with lamellar structure in the local tissue of the host, such zones are faded in the graft.

In these examinations there is an interesting parallel in the concepts concerning physiological growth (Frost 1963) reported above. Accordingly, the new growth of bone is the primary process and its resorption is a secondary one. *During periods of regeneration, the substitution of bone tissues is only the second part of a series of reactions* which start with processes of resorption. The type and quality of the bone tissue which is to be resorbed, determines the moment and the extent of bone substitution.

The question may arise as to what is the importance of the processes of resorption preceding the development of the callus in the sufficient and good regeneration of the bone tissue.

SUMMARY

Tetracyclines are deposited in the apposition zones of the compact and spongious substances and are strongly fixed by the mineralization process in the bone tissue.

A permanent rebuilding and regeneration process can be observed by tetracycline tracing.

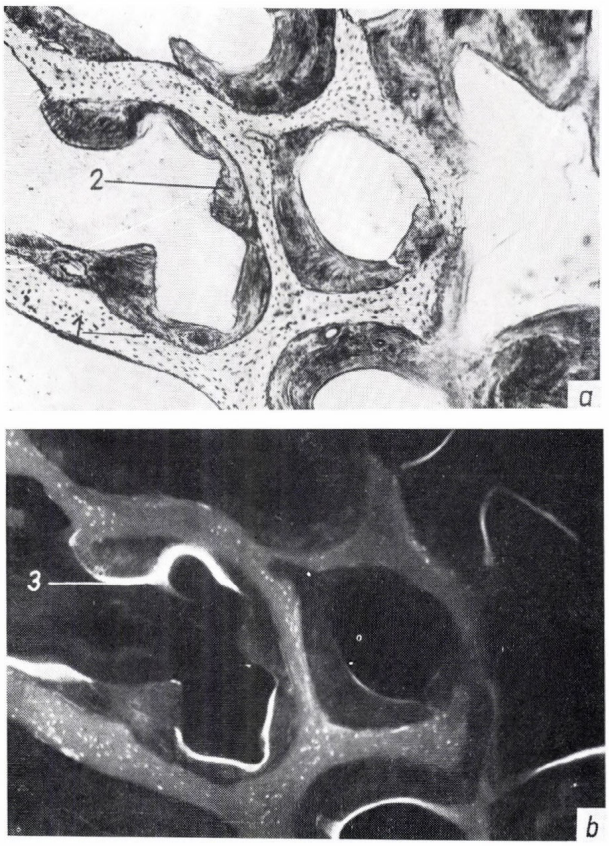


FIG. 9. Heterologous spongious transplant; a = in transmitted light; b = in ultraviolet light. Traced with tetracycline 3 days after resection. 1 = the dead graft; 2 = surrounded by the regenerating bone; 3 = fluorescent margins on the surface of the regenerating bone

The growth of regenerative bone tissue is preceded by the decomposition of the local bone: the latter process occurs differently in necrotic autografts and in heterografts.

A decomposed bone autograft is continuously substituted by fibrous bone, whereas a heterograft is surrounded by fibrous bone.

Fibrous bone will gradually change into lamellar bone tissue; it is fairly distinguishable as a regenerative bone by its special mosaic-like structure in polarized light.

The maturation of the fibrous bone was found to be inhibited by the uptake of tetracycline.

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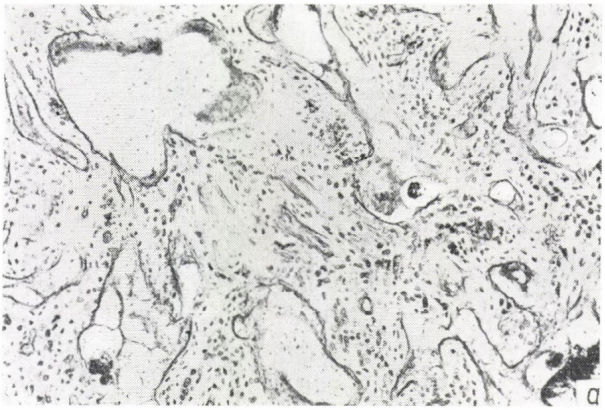

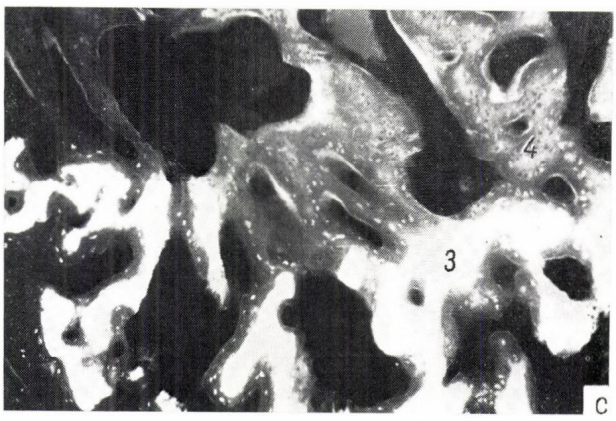

FIG. 10. Regeneration bone of callus traced with tetracycline about 3 months before resection; a = in transmitted light; b = in polarized light; c = in ultraviolet light. 1 = a younger regeneration bone already changed into short lamellar bone; 2 = fibrous bone traced diffusely with tetracycline 3 = showing fluorescence; 4 = slight fluorescence of the regeneration bone which derives mostly from the cavities of bone (osteocytes)

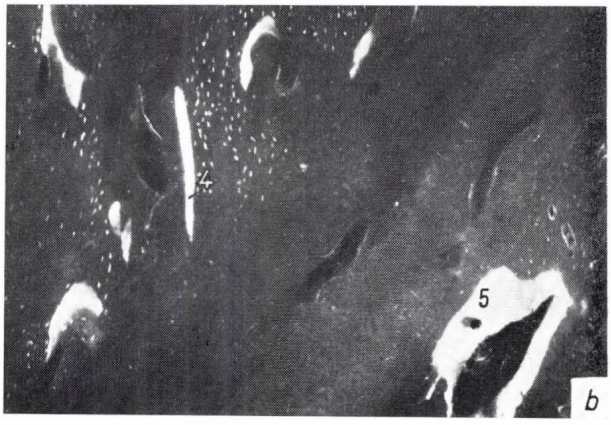

FIG. 11. Limit zone of a homologous transplant (splinter and fibula) and host tissue (radius); a = in polarized light; b = in ultraviolet light 2 years after its implantation. 1 = large amounts of lamellar bone of the host; 2 = the transplant has completely changed into short lamellar regeneration bone; 3 = border between host tissue and transplant; 4 = trace line in the host tissue or at the border; 5 = spot-like trace in the trans plant

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AUTORADIOGRAPHIC AND RADIOCHEMICAL STUDIES ON THE HEALING OF BONE FRACTURES

by

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Several methods are used for the quantitative and qualitative examination of the healing of bone fractures.

1. General morphological procedures, i.e. gross anatomical descriptions, measurement of circumference, examination of wet and dry weight, as well as water content, measurements of extension and strength, and the microscopic determination of the several stages of callus formation.
2. Special histological procedures for the analysis of metabolic processes during the healing of bone fractures, i.e. component and enzyme-histochemical methods for the demonstration of the synthesis of ground substance and fibrils as well as of the enzyme systems involved.
3. Biochemical procedures for better quantitative assay of synthetic products, especially by extractions of collagen and fractionations of mucopolysaccharides or by analysis of their components, i.e. uronic acids, hexosamines and hydroxyproline.
4. Procedures using radioactive isotopes are particularly suitable for comparative quantitative analyses of the synthesis and metabolism on small amounts of material, mainly for *in vitro* examinations of human and animal callus tissue.

In our examinations we preferred to use combined autoradiographic and radiochemical methods for the histotopological localization and simultaneous quantitative assay of synthetic processes using ^{35}S sulphate for the determination of neoformation of sulphated mucopolysaccharides and ^{3}H-proline for the analysis of collagen synthesis.

As has been shown by Lindner in his work on wound-healing (p. 35), the syntheses of ground substance and collagen may occur simultaneously in the same cartilage cell. As demonstrated by Lindner (1960b, 1961a, b, 1962 a, b, c, 1963, Lindner et al. 1962), in uninfluenced as well as influenced embryonic development of connective tissue, the formation of ground substance initiates before the formation of fibrils. Therefore, the synthesis of ground substance may be used as a measure of the development of connective tissue. Since, according to Gross et al. (1959) and others, the synthesis of mucopolysaccharide and protein moieties occurs jointly, we are able to obtain an approximate estimation of the synthesis of the whole mucopolysaccharide-protein complex. As the mucopolysaccharides of cartilage,

bone and callus consist preponderantly of chondroitin sulphates, the assay of the incorporation of ^{35}S sulphates can be employed as a quantitative method for the determination of ground substance and, eventually, of the neoformation of the connective tissue of cartilage, bone and callus (Lindner 1960a, b, 1961a, b, c, 1962a, b, c, 1963, Lindner et al. 1962, Becker et al. 1963, 1964, Köhler 1964, Schlieben 1964, Schlieben et al. 1964, Kröger 1965, Schröder 1965, Wittig 1965).

Investigations on the temporal connections of sulphation and synthesis of polysaccharides and the eventual exchange of sulphate groups, etc. were carried out by D'Abramo and Lipman (1957), Boström (1953), Boström and Aquist (1952), Boström and Mansson (1952), Boström and Odeblad (1953a, b), Boström and Roden (1961), Dziewiatkowski (1952, 1953, 1962), Amprino (1955a, b, c), Hilz (1960), Kennedy (1960) and others.

In our investigations on the callus, we measured the incorporation rates of ^{35}S sulphate after four hours of incubation *in vitro*, by which the synthesis and not the turnover of the chondroitin sulphates of cartilage, bone and callus can be determined. Thus, we did not estimate the specific activity of chondroitin sulphate separated by electrophoresis because, in this manner, not only the mere synthesis but the total turnover of chondroitin sulphate would have been determined as shown by comparative investigations (Becker et al. 1963, 1964). Moreover, the staining of separated chondroitin sulphate, according to Dittman and Cremer, needed for this procedure is disputable, for we have shown in our examinations that *in vitro* stain is not bound stoichiometrically, not even by pure mucopolysaccharides, and the procedure of Dittman and Cremer does not permit a quantitative separation of the chondroitin sulphates occurring in the tissue.

Thus, we have determined the incorporation rate of ^{35}S sulphate relative to the amount of tissue, i.e. to mg dry weight, by which, according to the above investigations, particularly those of Boström and Aquist (1952), Boström and Mansson (1952), Boström and Odeblad (1953a, b), Boström and Roden (1961), Dziewiatkowski (1952, 1953, 1962), Schiller et al. (1956), Kennedy (1960), Belanger (1956), Kowalewski (1958a, b), Layton (1951a, b, c) and those of our working team, the synthesis of chondroitin sulphate and so the synthesis of ground substance can be determined. Since the investigations of Krompecher (1956, 1958) this subject has come to the foreground of interest.

In conformity with other authors, Lindner and co-workers found that in embryonic and postembryonic development of cartilage and bone, biochemical differentiation precedes morphological differentiation. We know the primary concentration of acid mucopolysaccharides which may be demonstrated histochemically with appropriate staining methods in cartilaginous blastemata of 6-day-old chick embryos (Fig. 1), even before further cellular differentiation. The same finding was obtained also in autoradiographs after application of ^{35}S (Fig. 2). Figures 1 and Fig. 2 demonstrate different contents of sulphated mucopolysaccharides with varying degree of esterification of unequally differentiated cartilaginous blastemata by modified Astra blue staining (Fig. 1) and in autoradiogram (Fig. 2) after incorporation of ^{35}S with corresponding different blackening intensity. Less known

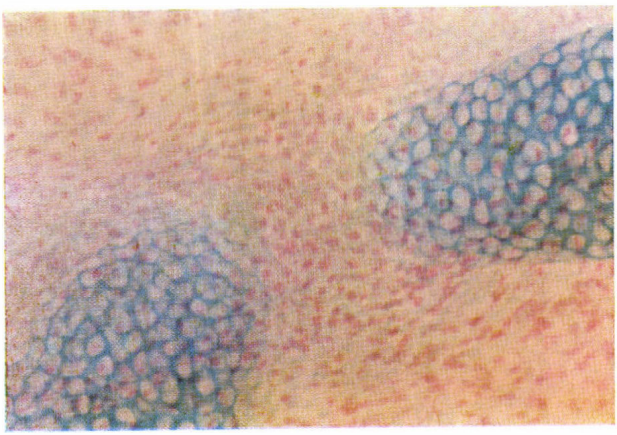

FIG 1. Demonstration of different content of sulphated mucopolysaccharides with different degree of esterification of unequally differentiated cartilaginous blastema with modified astra-blue staining

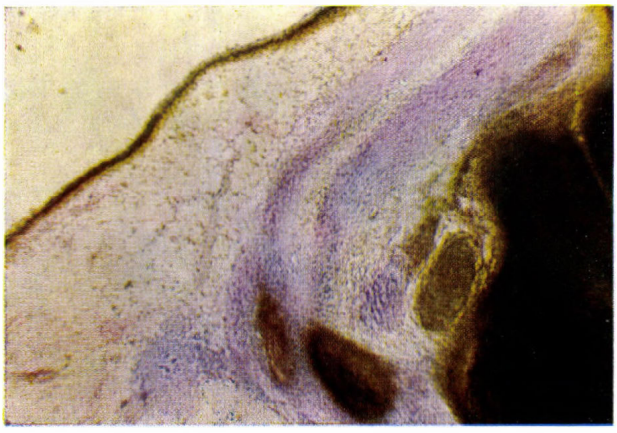

FIG. 2. Autoradiogram after incorporation of ^{35}S with corresponding different blackening intensity. As is known, biochemical differentiation precedes morphological differentiation

is the fact which will be demonstrated in the following that this primary biochemical differentiation takes place during further development of bone as well. As far as we know, this is the first morphological demonstration of this process.

Figure 3 shows the metachromatic reaction with toluidine blue in the ^{35}S-autoradiogram. The blackening produced by ^{35}S containing material, exceeds the strictly limited zone of metachromasia marked with arrows, indicating the precise localization of polysulphuric esters of the cartilage ground substance having high molecular weight. Obviously, they are compounds containing ^{35}S with no high molecular weight which, in the growth margins of the cartilaginous blastema, are probably made available for the sulphation of cartilage polysaccharides.

According to these examples, it is possible to demonstrate autoradiographically with special methods of double labelling (see p. 35) the for-

FIG. 3. Metachromatic reaction with toluidin blue on the ^{35}S autoradiogram: the blackening produced by ^{35}S exceeds the strictly limited zone of metachromasia marked with arrows which shows the precise localization of high-molecular weight polysulphuric esters of the cartilaginous ground substance

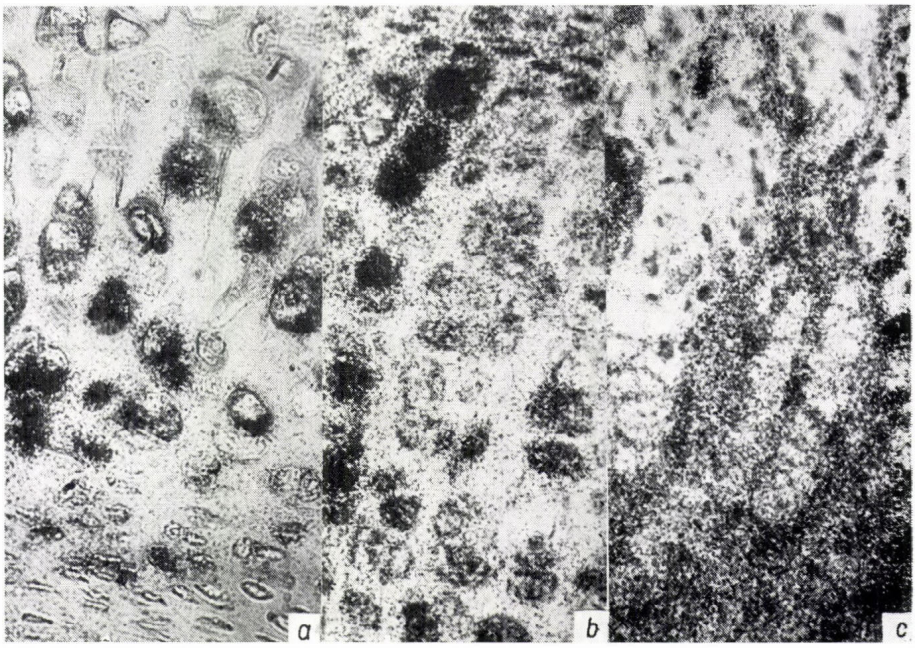

FIG. 4. Sulphation and synthesis of acid mucopolysaccharides in chondrocytes during the first hour of ^{35}S incorporation (a). Decrease of ^{35}S activity over the cartilaginous cells and increase of ^{35}S activity over the intercellular substance 24 hours after ^{35}S incorporation (b). End of extracellular release of labelled mucopolysaccharides; the cartilaginous cells are unlabelled after several days of ^{35}S incorporation (c)

mation of ground substance in cartilage and bone cells like in other connective tissue cells, as well as the simultaneous formation of ground substance and fibrils in the same cartilage cell (Lindner 1963, Schmidt et al. 1963).

In conformity with Dziewiatkowski (1962), Boström (1953), Belanger (1956) and others, we examined systematically the temporal course of intracellular mucopolysaccharide synthesis and that of the release of labelled mucopolysaccharides into intercellular space. This temporal course is demonstrated in Fig. 4.

Even 30 to 60 minutes after a single application of ^{35}S, essentially the same processes are observed in different experimental animals and in different cartilage tissues as demonstrated on the rib cartilage of the rat in Fig. 4. This applies, apart from slight differences, to the further temporal course as well. During the first hour, processes of incorporation and anabolism are already demonstrable in the chondrocytes, i.e. sulphation and synthesis of acid mucopolysaccharides in the chondrocytes can be demonstrated morphologically (Fig. 4a). During the further course, up to 24 hours, substantial radioactivity and autoradiographically demonstrable blackening remained over the cytoplasm of the cartilaginous cells, by which, according to the corresponding evidence presented by Dziewiatkowski (1962), the intracellular synthesis of mucopolysaccharides has been demonstrated. After 24 hours, radioactivity was found to decrease over the cartilaginous cells and increase over the intercellular substance (Fig. 4b). The same situation is observed in the following days, until, finally, depending on the experimental conditions as well as on the localization and age of the examined cartilage, no labelling is displayed by the cartilage cells. The extracellular release of labelled mucopolysaccharides has come to an end (Fig. 4c).

Therefore, in agreement with former statements, after ^{35}S incorporation of a four hour duration the intracellular synthesis of sulphated acid polysaccharides is demonstrated. This holds for incorporation *in vivo* as well as *in vitro* (on the analogy of the investigations of Dziewiatkowski [1962] and others). Similar findings have been obtained in the callus in our preliminary comparative morphological and radiochemical examinations.

In our experiment inbred Wistar rats of a strain owned by the Institute were used to ensure uniform and reproducible material. The following procedures and examinations were performed: mechanical production of closed femoral fractures, comparative morphological, particularly autoradiographical examinations (histochemical procedures included) for the control of the normal course of fracture healing, combined with quantitative radiochemical examinations. The purpose of our examinations was to clarify:

1. whether the measurement of the incorporation rate of ^{35}S does reflect the temporal course of fracture healing with a corresponding curve having a clear maximum at the greatest neoformation of sulphated mucopolysaccharides;
2. whether drugs or other substances acting on the connective tissue, generally used in fracture healing in human medicine, will delay, accelerate or cause any qualitative or quantitative changes (e.g. by depression of the maximum by temporal shifting or broadening of the normal maxi-

mum) in the normal temporal course of the formation of mucopolysaccharides and simultaneously that of the callus;

3. whether the influences mentioned under 2 are or are not dependent on concentration, since it has been shown (Lindner 1959, 1960 a, b, 1961a, b, 1962b, c, 1963, Lindner et al. 1962) that drugs depending on their concentration may have antagonistic effects on embryonic or postembryonic development of connective tissue which fact is of great clinical importance.

Systematic examinations were performed first in dosage series on *Cortisol* and *phenylbutazone*, as well as on its principal metabolite: *oxyphenbutazone*. Then concentration series were carried out on dextran-containing plasma expander *Rheomacrodex* and on *Liquemin* (a heparin preparation).

Treatment of intraperitoneal injections twice daily was begun on the day of fracture induction and carried on until 24 hours preceding the animals' sacrifice. After the animals were killed, the callus tissue was isolated, immediately reduced to pieces under appropriate conditions, transferred to Warburg flask and incubated for four hours in Krebs-Ringer-phosphate or Krebs-Ringer-substrate buffer solutions with 1 or 5 μc of ^{35}S added. In order to obtain comparative data, cartilage tissue of rib and xiphoidal processus, intact femoral bone as well as skin of the back and aortae of treated and untreated rats were incubated in the same manner. During incubation in the Warburg apparatus, oxygen consumption was measured constantly in the usual way and subsequently calculated with regard to the wet weight determined previously or to the dry weight determined subsequently.

The incubation of four-hour duration was followed by dialysis lasting 24 hours, in order to eliminate the non-incorporated inorganic sulphate. Then the following procedures were performed: determination of dry weight, wet ashing, precipitation of sulphate as barium sulphate, measurement of radioactivity in methane-flow-counter (Frieseke and Hoepfner) and recording of the results as impulse rates (min/mg dry weight; for further details see Lindner 1962 a, b, c, Lindner et al. 1962, Schmidt et al. 1963, Becker et al. 1963, 1964).

The comparative autoradiographic examinations, mentioned above, were carried out with the stripping film technique using Kodak AR 10, and the accompanying histochemical examinations were performed using component-histochemical procedures, particularly for the demonstration of acid mucopolysaccharides.

The results of morphological estimations are not reported here, since the gross anatomical and microscopical patterns (obtained with routine staining methods) of callus formation, and that of the healing of these experimental fractures of the rat femur are known. Our findings have been in full agreement with those obtained by other authors.

From these examinations an important result was obtained, namely that by histochemical methods the period from the ninth to the fourteenth day after fracture has been established as the period in which the acid mucopolysaccharide content of the callus tissue is the highest.

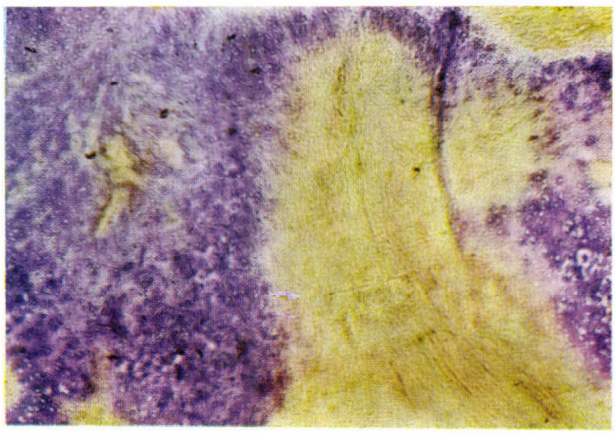

FIG. 5. High content of acid mucopolysaccharides (violet) demonstrated by toluidine blue in comparison with neutral polysaccharides (red) in the callus tissue of 11-day-old femoral fracture of rat

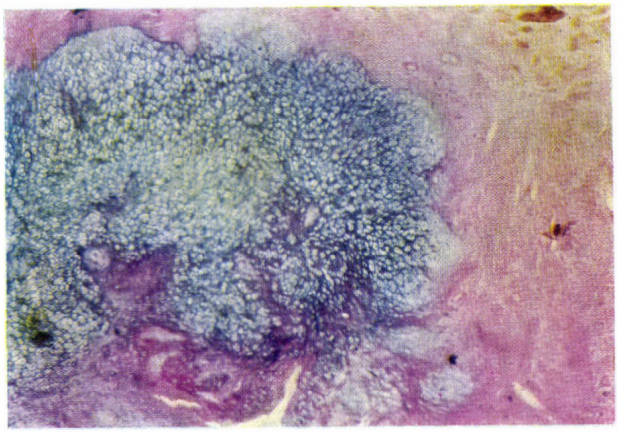

FIG. 6. High content of acid mucopolysaccharides (blue) demonstrated by combined astra blue—PAS staining in comparison with neutral polysaccharides (red) in the callus tissue of 14-day-old femoral fracture of rat

Figures 5 and 6 show the high acid mucopolysaccharide content violet (Fig. 5) or blue (Fig. 6), demonstrated by toluidine blue or combined Astra blue—PAS staining, in comparison with neutral polysaccharides (red) in the callus tissue of 11-day-old (Fig. 5) and 14-day-old (Fig. 6) femoral fractures of rats.

By parallel autoradiographic examinations, as mentioned above, an identical histotopological localization of ^{35}S incorporation was established in the chondroblasts of callus tissue after incorporation *in vitro*, and in the chondroblasts of growing cartilage after incorporation *in vivo* (Fig. 4). This proves the fact that, during an incubation *in vitro* of four hours with ^{35}S, this substance is employed almost exclusively for the intracellular synthesis of acid mucopolysaccharides.

In Fig. 7 the intracellular incorporation of ^{35}S into the chondroblasts of 12-day-old (Fig. 7a) and 14-day-old (Fig. 7b) callus tissue is demonstrated.

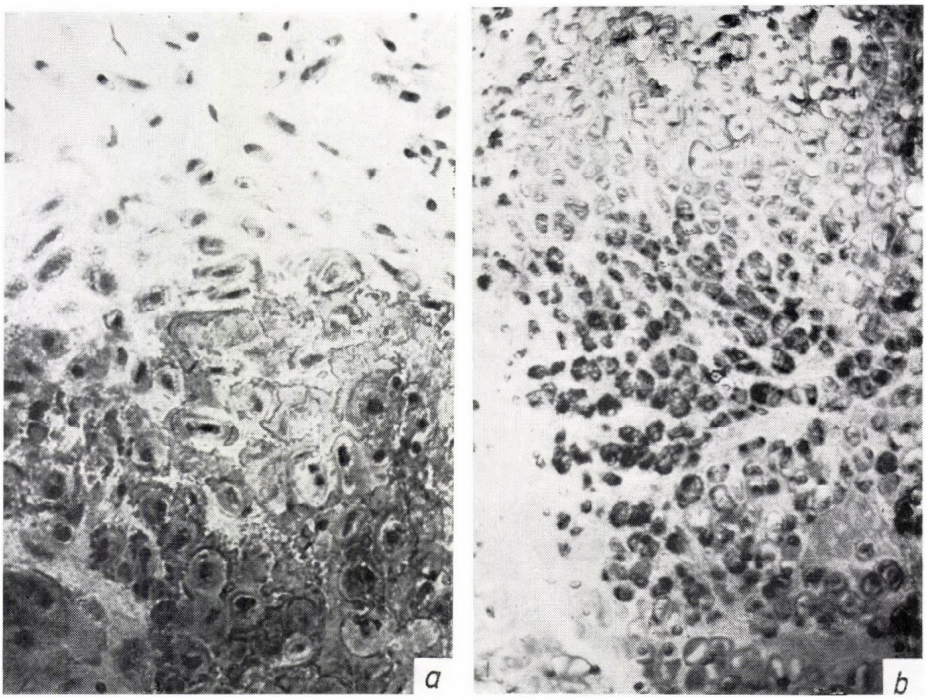

FIG. 7. Intracellular incorporation of ^{35}S in chondroblasts of 12-day-old (a) and 14-day-old (b) callus tissue

By the radiochemical examination of this material we have been able to demonstrate the synthesis of acid mucopolysaccharides and perform quantitative determination of this synthesis in comparative experimental series.

Figure 8 shows that under different conditions in the tissues mentioned, oxygen consumption is parallel with the incorporation of sulphate and thus, with the synthesis of mucopolysaccharides. On the left of the figure it is demonstrated that under equal experimental conditions the untreated costal cartilage has the highest oxygen consumption. Previous treatment with 10 mg hydrocortisone/200 g rat, daily for 16 days was found to decrease oxygen uptake precisely as it decreased the uptake of ^{35}S. In comparison with young animals weighing 200 g, usually employed by us, the rib cartilage of older animals showed a still smaller extent of oxygen consumption. The smallest degree of oxygen consumption was observed in the rib bones and back skin of untreated controls. All these values are averages. The right side of Fig. 8 illustrates that pretreatment of the rats with hydrocortisone for 16 or 14 days, results in a reduction of oxygen consumption depending on the dose (5 or 10 mg/200 g rat daily), while a 12-day treatment with 2·5 mg oxyphenbutazone/200 g daily stimulates the oxygen consumption of the callus tissue compared with that of the controls. The same holds for the incorporation of sulphate.

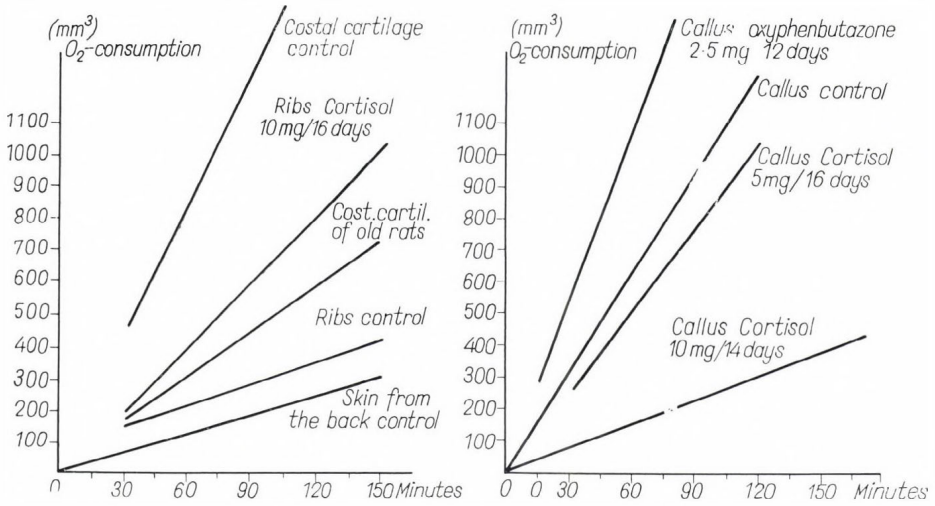

FIG. 8. O_2-consumption by cartilage, bone and skin. Comparison of the influence of Cortisol and oxyphenbutazone with untreated controls

Figure 9 shows a section of the normal course of the synthesis of callus ground substance, with a maximum between the tenth and the thirteenth day after experimental femoral fracture of the rat. In addition, a remarkable stimulation of mucopolysaccharide synthesis in the callus is recognizable on the left side, indicating the averages of the incorporation rate of xiphoidal process, rib cartilage and rib bone, compared with intact cartilage and bone tissue. Finally, from Fig. 9 it is clear that the three doses of Cortisol used (1, 5 and 10 mg/200 g rat daily, i.e. 5, 25 and 50 mg/kg rat daily), compared with the controls, resulted in a marked inhibition of the ^{35}S-incorporation and thus, of the formation of ground substance and callus, parallel with the inhibition of oxygen consumption. The maximum of ^{35}S-incorporation and mucopolysaccharide synthesis in untreated calluses between the tenth and thirteenth day after fracture was found to be remarkably reduced or even absent following Cortisol treatment.

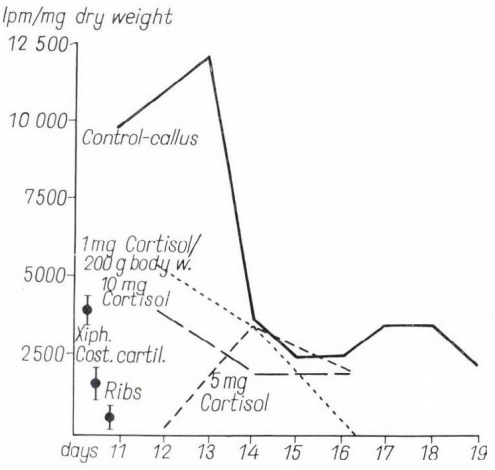

FIG. 9. Comparison of the normal course of the synthesis of callus ground substance with the influence of Cortisol (different doses) upon ground substance synthesis

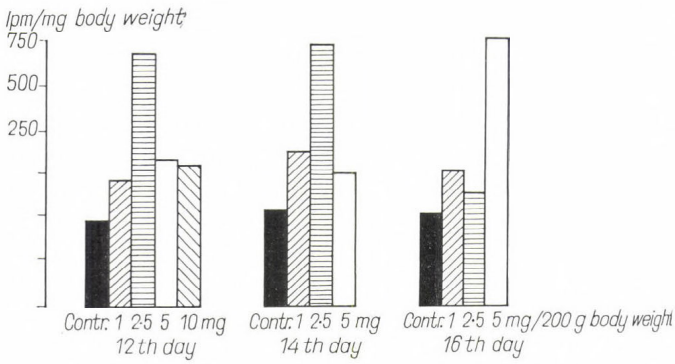

FIG. 10. Influence of oxyphenbutazone (different doses) upon ground substance synthesis. Comparison with untreated controls

These findings are in agreement with the corresponding results of other authors also obtained with ^{35}S in normal mucopolysaccharide synthesis and in that influenced by Cortisol treatment. Mention should be made of the work of Layton (1951a, b, c) on intact cartilage and bone of rats, Boström and Odeblad (1953a, b) Schiller et al. (1956), Huble (1957) on chick embryo, Kowalewski (1958a, b) on fracture callus, Lash and Whitehouse (1961) on chondrogenesis, etc. The results of these authors are in agreement with those obtained by us (Lindner 1961a, b, 1962 a, b, c, 1963, Lindner et al. 1962, Becker et al. 1964), Schlieben (1964) Schlieben et al. (1964), Kröger (1965) and Wittig (1965). The hormonal influences on the healing of bone fractures, emphasized by Krompecher (1958), have been kept in view also in therapy.

The findings on the influence of Cortisol and some of the drugs on embryonic and postembryonic formation of connective tissue are discussed on p. 35

According to these examinations, the effect of substances does not depend only on time but also on concentration, so that increasing concentrations can result not only in linear curves of inhibition or stimulation of connective tissue, but also in contrary effects. The embryonic development of cartilage and bone was particularly evidenced by Kröger (1965), and by other authors, e.g. by Hilz (1960) in experimental atherosclerosis of the rat, by Rohde et al. (1961) in cultures of fibroblasts, etc. These findings are important clinically. Similar findings were obtained in our examinations on callus.

Figure 10 demonstrates that the treatment of bone fractures with oxyphenbutazone produces an increased synthesis of ground substance, unequivocally depending on concentration.

The rats were given 1, 2 · 5, 5 and 10 mg/200 g rat daily, i.e. 5, 12 · 5, 25 and 50 mg oxyphenbutazone/kg rat daily. This daily dose was administered in two intraperitoneal injections, beginning from the day of fracture induction. From Fig. 10 it is clear that the medium dose of 2·5 mg/200 g rat is the most effective in stimulating the incorporation of ^{35}S and thus, the synthesis of mucopolysaccharides, by which the neoformation of connective tissue—in the present case, that of callus—can be quantitatively determined. The

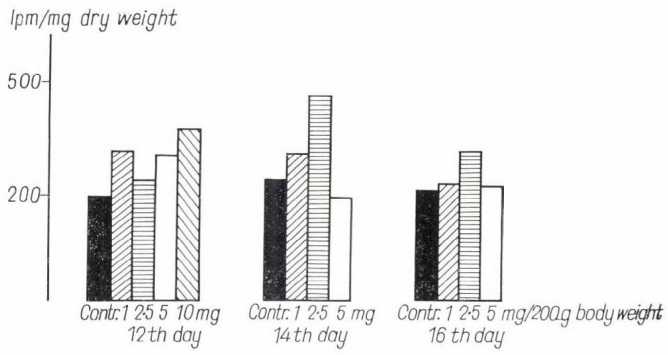

FIG. 11. Influence of phenylbutazone (different doses) upon ground substance synthesis. Comparison with untreated controls

callus was found to increase with other concentrations of oxyphenbutazone, too, compared with the controls. It is of interest that the effect of oxyphenbutazone was recognizable even gross anatomically by the condition and extent of the callus tissue, microscopically, and in particular by histochemical evidence. It is not possible, however—in opposition to the radiochemical methods used by us for this purpose—to obtain precise quantitative data with this procedure (as well as with ^{35}S autoradiograms).

It is also of interest that the same concentrations of the mother substance, phenylbutazone, also showed an increase of the mucopolysaccharide synthesis of the callus (Fig. 11), though not identical with oxyphenbutazone and merely on the twelfth day with the doses examined. On the fourteenth day the increase is greater with daily doses of 1 or 2·5 mg/200 g rat (i.e. 5 or 12·5 mg/kg body weight), whereas doses of 5 mg/200 g rat (i.e. 25 mg/kg body weight) effect a decrease, compared with the controls. On the sixteenth day the medium dose alone results in higher values than in the untreated controls, while the values obtained with the other doses are not essentially different from those of the controls. It is again the medium dose that mostly exceeds the control values on the fourteenth day, too. Eventually, the values of phenylbutazone obtained with similar doses are not essentially less than those of oxyphenbutazone, though the double amount of the most active metabolite of phenylbutazone has been applied during the treatment of oxyphenbutazone. This finding is an indication of the fact that not only oxyphenbutazone, the first metabolite of phenylbutazone, but also phenylbutazone as a whole, and probably its other metabolites seem to have a specific influence on the neoformation of connective tissue (Lindner 195), 1960a, b, 1961a, b, 1962a, b, c, 1963, Lindner et al. 1958, 1962, Eckstein et al. 1960, Schweinitz et al. 1960, Schlieben 1964, Gries 1965, Schröder 1965, Wittig 1965, Kröger 1965).

As a result of our comparative morphological (histochemical and autoradiographical) as well as radiochemical examinations on experimental fracture healing in the rat, the following conclusions have been drawn.

As evidenced in preliminary examinations, the incorporation rates of ^{35}S *in vitro*, after an incubation of 4 hours in Warburg apparatus (measuring

simultaneously oxygen consumption), can be used as a quantitative measure of the synthesis of acid mucopolysaccharides.

After the histotopological localization of ^{35}S-incorporation in embryonic and juvenile cartilage, and in callus tissue, evidence of intracellular incorporation of ^{35}S and mucopolysaccharide synthesis and morphological proof and control of the radiochemical examinations are reported.

During normal callus formation, maximal mucopolysaccharide synthesis is found between the ninth and thirteenth day after fracture.

In order to elucidate the theoretical and practical importance of whether this maximum and, generally, the course of mucopolysaccharide formation (as decisive synthetical step of the healing of bone fractures) undergo qualitative or quantitative changes due to delaying or accelerating influence of drugs, the effect of certain drugs used in human medicine during healing of bone fractures were analysed.

The most important result of these examinations appears to be the finding that the influence of these drugs on callus formation (similar to other connective tissues) depends on concentration in a special manner, i.e. contrary effects may occur with increasing concentrations. This finding is of great theoretical and practical importance.

At the same time, these examinations have shown that by comparative morphological and radiochemical methods it is possible to localize histotopologically and obtain precise quantitative determinations concerning the decisive steps of intracellular and extracellular formation of connective tissue.

Finally, the comparison of these findings with the results obtained in other connective tissues shows similarities, but also peculiarities in the synthesis and metabolism of the various connective tissues leading thus, to further progress in the systematical investigation of connective tissues under physiological and pathological conditions.

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THE ORIGIN OF THE PERIBLASTEMA AND ITS ROLE IN CALLUS FORMATION

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Manuscript not received.

Symp. Biol. Hung., 7, pp. 205—215 (1967)

FURTHER OBSERVATIONS ON BONE ELONGATION BY ANDERSON'S METHOD

by

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At a meeting of the Polish Orthopaedic and Traumatic Association held in Lublin in 1963, we reported on our early observations on bone elongation in lower extremities of children in postpoliomyelitis state, treated by Anderson's (1952) method which we introduced in 1961. This preliminary clinical report was based on the analysis of 10 cases. So far we have treated 65 cases. Apart from several cases where the children are still kept in plaster following operation, and it would be too early to assess these results, we have analysed 50 cases in which the period of observation ranged from 1 to 4 years. The apparatus employed for elongation is presented in Fig. 1.

During four years of study (Mitchell 1963, Trueta 1956) and work we have introduced certain modifications in the procedure adopted, and we have also encountered some complications and difficulties which are discussed, so that orthopaedists interested in this method could avoid them.

1. To shorten the time needed for elongation of the extremity, we have introduced a modification which consists of the simultaneous performance of the first stage surgery, i.e. resection of the fibula right above the ankle and fixation of its lower part to the tibia with a nail or a wire loop; the main operation consists in the subperiosteal fracture of the tibia half way its length and subsequent elongation in Anderson's apparatus. By this modification we have been able to perform the two operations in one stage and have gained six weeks which had to elapse between the two operations by using the original method.

2. Serious difficulties were encountered in maintaining the initial position of the foot as the elongation progressed. The triceps muscle being the strongest of the leg muscles caused equinus foot, a still larger deformity (22 cases) which in 2 patients necessitated a second procedure of correction (Figs 2 and 3). At first plantar flexion was ensured by a posterior plaster trough, and then by an U shaped plaster support with its ends fastened to the apparatus. In some cases this exerted a pressure on the upper part of the ankle, flattening the foot even bringing about a deformity (1 case, Fig. 4).

To avoid these complications, about one year ago we have introduced fixation of the ankle in fastening the heel by two Kirschner wires, to the tibia. Satisfactory results were obtained and the damage caused in two small points of the articular cartilage was much smaller than the effect

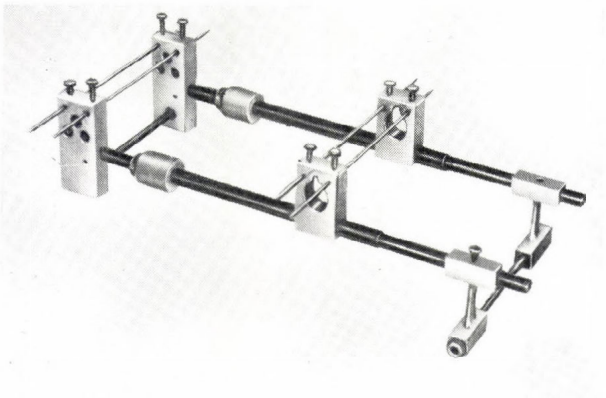


FIG. 1. Extension apparatus used for elongation of the extremities

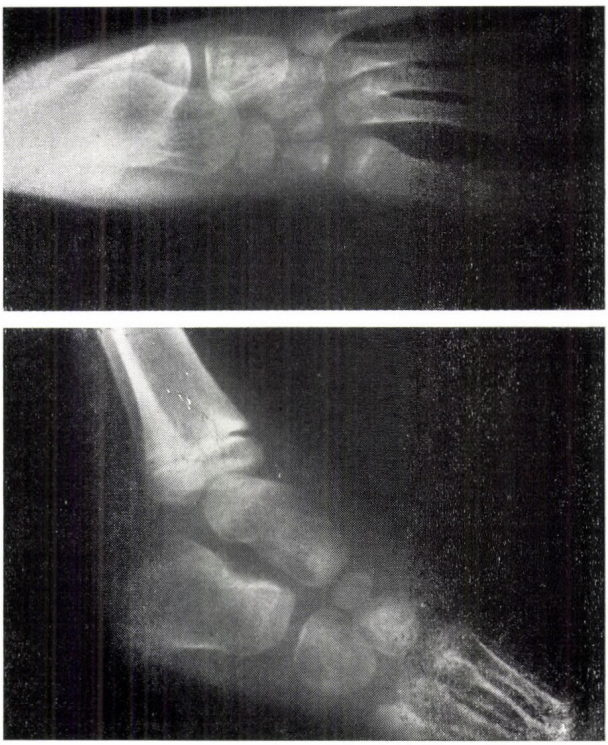


FIG. 2. Secondary deformity (equinus foot) caused by pressure of the triceps muscle

of a prolonged and ever increasing pressure upon the articular surfaces (Fig. 5).

We have also adopted the principle that in case of equinus foot, prior

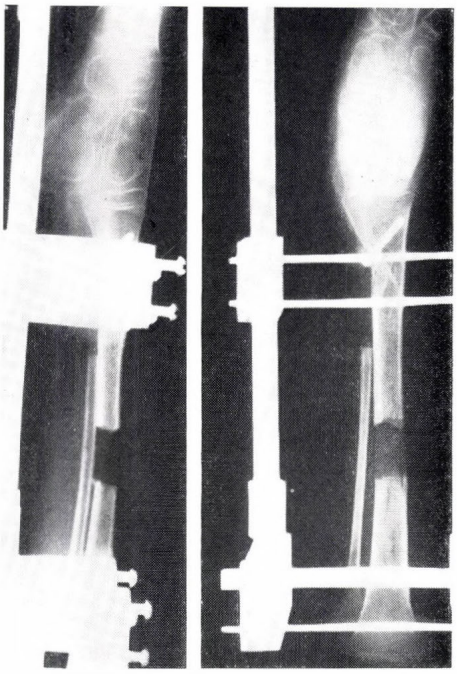


FIG. 3. Procedure to correct a secondary deformity

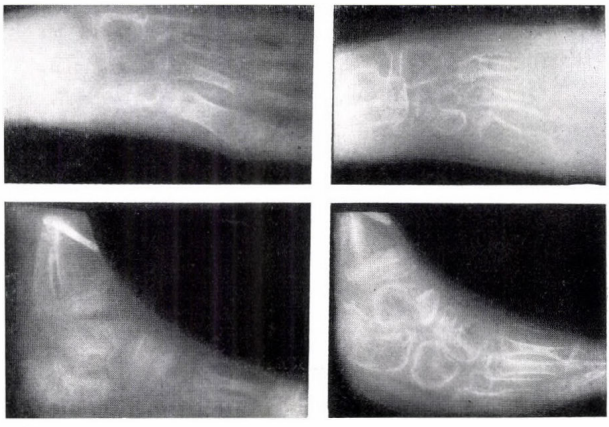


FIG. 4. Deformity caused by the pressure of the apparatus on the upper part of the ankle

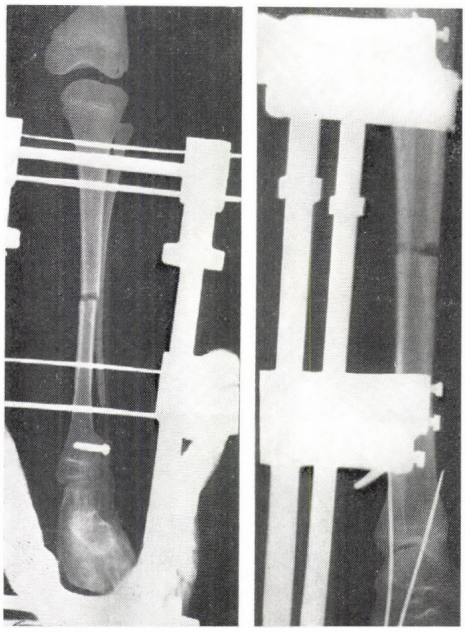


FIG. 5. Fixation of the ankle by fastening the heel with two Kirschner wires to the tibia

to the main operation we elongate the Achilles tendon. The initial positioning of the foot should be 90°.

3. An increased tension of the Achilles tendon also resulted in angular dislocation of the fragments, particularly in the plane of the fibula (Fig. 6). In time, however, such dislocations healed well and the axis of the leg became gradually compensated (Fig. 7).

4. A too low introduction of Steinman's pins near the ankle resulted in pain, swelling and limitation of the range of foot mobility. This complication was avoided if the bolts were placed about 5 cm above the joint (1 case, Figs 8 and 9).

5. If the extremity in plaster was loaded too early, it resulted in an inflection of the newly formed bone by approaching the fractured bone ends towards each other (3 cases, Fig. 10). This causes loss of elongation already achieved and it retards healing. Loading of the extremity in plaster follows now, depending on the X-ray picture giving evidence of the formation of fresh bone tissue. Generally, this occurs 4 to 5 months after the process of elongation has come to an end, i.e. one month in the apparatus, two months in plaster with two Steinman's pins (one upper and one lower), to counteract muscular forces, and one month in plaster dressing without load. The plaster dressing can be removed, but full loading can only be started after the X-ray picture shows evidence of the presence of the bone-marrow canal and a cortical bone layer (Figs 11 and 12). This is generally attained after 8 to 10 months.

6. In 2 cases (Fig. 13) fracture of the elongated part occurred as a result of a traumatic injury. Both cases healed well (Fig. 14).

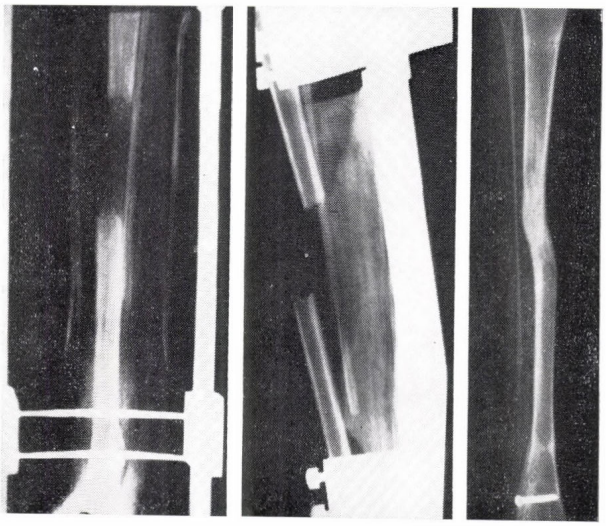


FIG. 6. Increased tension can result in angular dislocation of the fragments (see also Fig. 7)

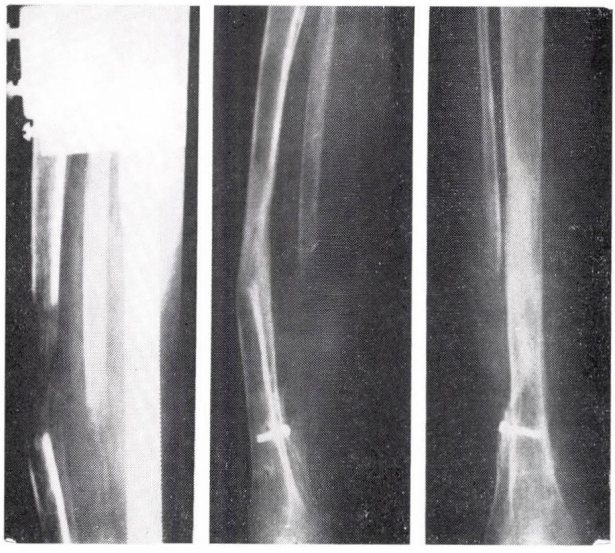


FIG. 7. Angular dislocation as a result of treatment (see Fig. 6) can be gradually compensated

In children aged 13 to 15 years, we have performed double fracture of the tibia in order to diminish the distance between the bone ends. The rate of healing was normal in 5 cases (Figs 15 and 16).

7. Minor superficial reactions around Steinman's pins were checked with pads soaked in Rivanol. The gauze placed around the bolt close to the skin surface was kept wet. We have obtained good results with open treatment applying Genciana on the skin, without covering the bolts with gauze. The reaction

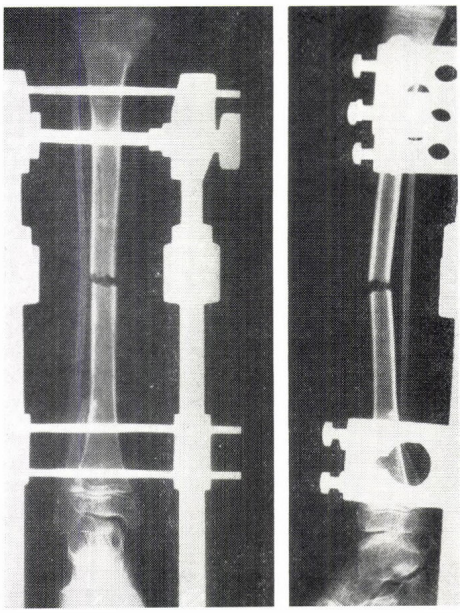


FIG. 8. Steinman's pins located too low caused pain, swelling, etc. (see Fig. 9)

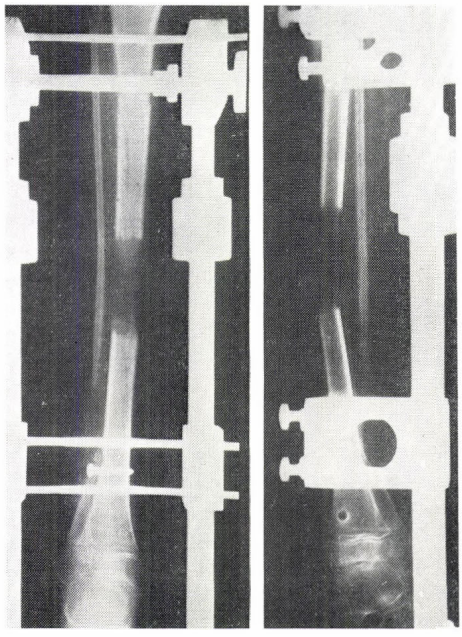


FIG. 9. Undisturbed further treatment was achieved by placing the bolts about 5 cm above the ankle joint

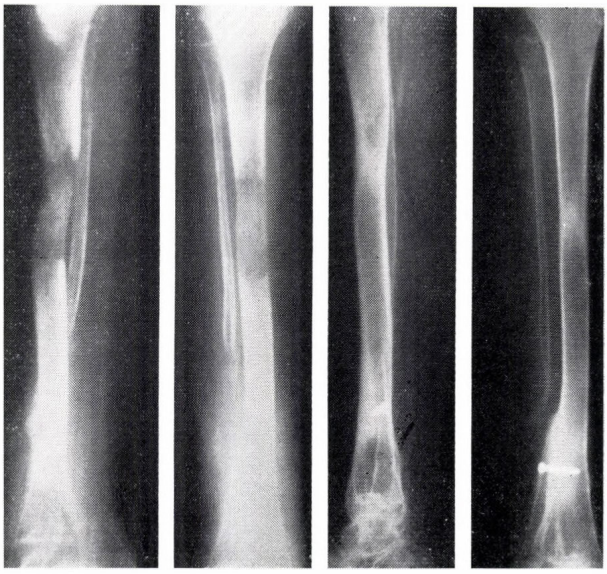


FIG. 10. Too early loading of the elongated bone results in an inflection of the newly formed bone causing loss of elongation and retarded healing

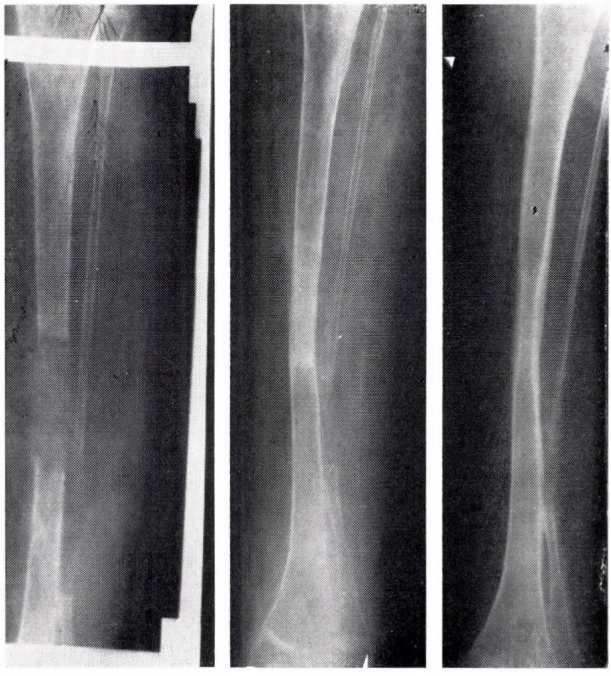


FIG. 11. Right time of loading determined by the X-ray picture; appearance of a cortical layer

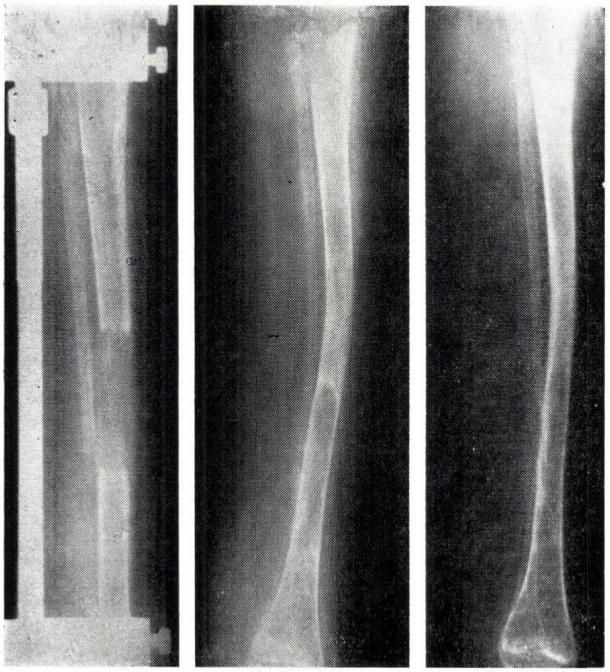


FIG. 12. See caption to Fig. 11

was generally superficial. Only in one case have we encountered more serious reaction: pain, rise of temperature, etc. In this case elongation had to be terminated, the pins and the apparatus removed, though only an elongation of 4 cm had been obtained instead of the planned 6 cm. After removal of the apparatus and pins, healing was completed in four weeks.

TECHNIQUE

First step. A longitudinal incision on the anterolateral surface of the leg made on one-third of its lower part permits to reach the fibula. Then the periosteum is incised and removed, and an oblique cut is made through the bone externally upward and internally in a sagittal plane. From the upper part of the bone a piece of about 1 to 2 cm is cut off. After the periosteum has been removed, a slanting incision is made across the tibia by which a bone wedge is formed. The lower part of the fibula is inserted under this wedge. Then plaster was put on for six weeks. Such a procedure prevents external rotation of the foot when the leg is later exposed to extension.

Main operation. Four Steinman pins (two upper and two lower) are passed through the holes in the extension apparatus in the epiphyseal region of the tibia in a frontal plane. The apparatus is removed: a small incision is made in the skin and the tibia is drilled through transversally at half its length,

FIG. 13. Fracture of the elongated part due to traumatic injury

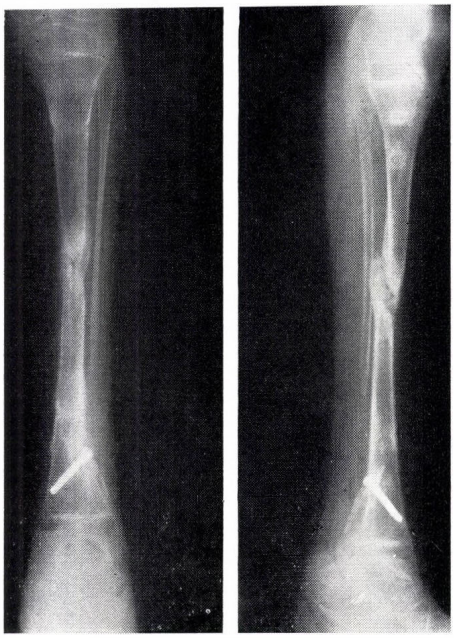
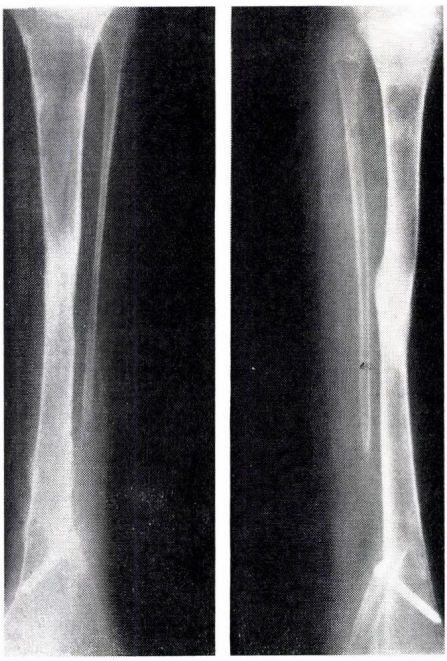


FIG. 14. Healing of fracture of the elongated part (see Fig. 13)



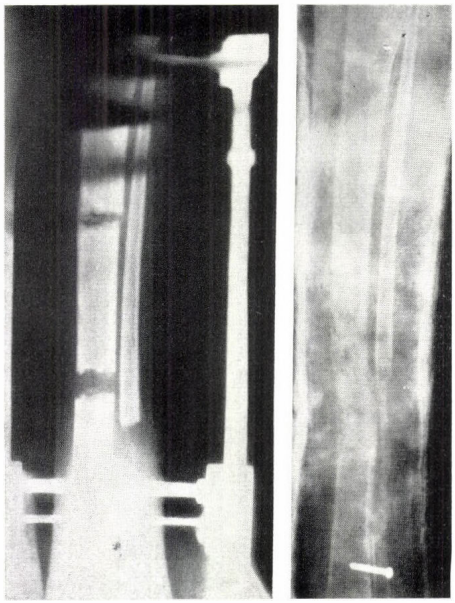


FIG. 15. In children aged 13 to 15 years, double fractures were made on the tibia to diminish the distance between the bone ends. Normal healing

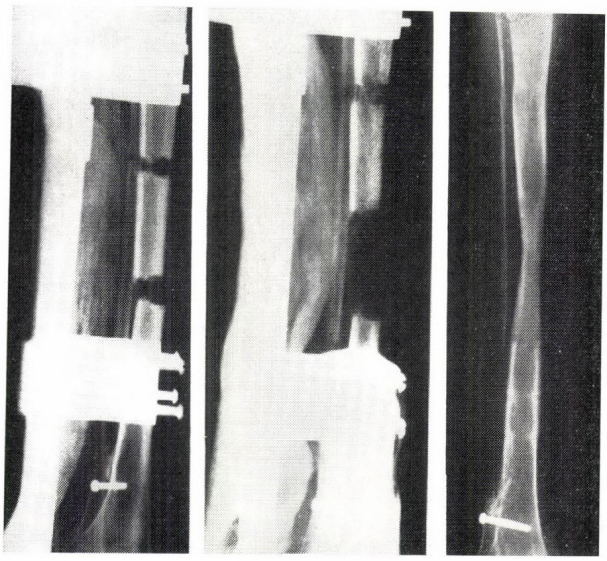


FIG. 16. See caption to Fig. 15

and the shaft of the tibia is broken at this level. The extension apparatus is again placed on Steinman pins and the bone ends are fixed. A plaster splint may be used to support the foot.

Procedure. The day after the operation the process of elongation is started by means of the distance-adjusting screws on the apparatus and they are given a half turn twice a day. The screws are calibrated and a 180° turn provides an elongation of 0·5 mm (one full turn daily, i.e. 1 mm). The elongation process is checked by periodic X-ray examinations. When the desired length has been obtained, the extremity remains in the apparatus until the fresh bone tissue becomes adequately calcified in about ten weeks. If the apparatus were to be removed too early, this would result in diminishing the elongation obtained because of the contracting effect of the muscles. After removal of the apparatus, the extremity is placed in plaster until the bones are entirely firm.

CONCLUSIONS

As a result of the analysis of 50 cases, we believe that Anderson's procedure is the method of choice in elongating the extremities in children where length loss does not exceed 3 to 4 cm.

In applying this technique, particular attention should be paid to have the Achilles tendon set under proper tension and to the position of the foot as well as to its fixation at an angle of 90°.

Loading of the extremity should be started while it is in plaster and under X-ray control, though not earlier than four months after completion of the elongation process. Full loading without plaster dressing can be performed, depending on the X-ray pictures, but not earlier than after eight months.

As regards children up to 12 years of age, we believe that there is no apparent justification to limit the range of elongation to 6 cm. In several cases we have obtained elongations ranging from 12 to 14 cm. In such cases the time of immobilization has been prolonged.

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Symp. Biol. Hung. 7, pp. 217—225 (1967)

EFFECT OF FUNCTIONAL FACTORS ON CALLUS FORMATION IN CLINICAL PRACTICE

by

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IN THE healing of bone fractures, the demonstration of the role of function and its systematic employment is but a part of our comprehensive scientific subject in which the functional reaction to stimulus and the correlations of tissue regeneration are investigated in the treatment of injured people.

In its present formulation, the qualitative adaptation and the systematic use of the specific biological stimulus as a healing factor as conceived by Krompecher provides the theoretic basis for modern functional treatment.

The application of the method is just as varied and diverse as are the various kinds of fractures and their degree of severity.

In this study I wish to present the experience obtained in the least solved but all the more discussed field of this problem, namely, the experience gained in the course of *the operative and immediate postoperative functional treatment of bone fractures.*

The task was outlined in Riga by Klemm in 1894 as follows: the bone must be assured of *rest,* but the joints, the muscles, the tendons, the fascia must not be deprived of *movement.*

This concept is reflected by Küntscher's classical operative method as well as by the procedure of Willenegger and Schenk.

The contrast between movement and rest in the identical segments of the organism can only be resolved if the injured organs are structurally restored. The purpose of the method is to disregard external fixation entirely or to modify it in such a way as to enable all the joints of the extremity to function at least during the period of treatment. This provides the only possibility for the medium tissue of the ruptured tissue structure to differentiate subsequently into valuable elements under the influence of the functional stimulus and prevent it from developing into a scar which would mean failure of regeneration.

Numerous authors are prone to blame the operation directly for the cicatrization and the insufficient callus formation.

Our experience, however, contradicts this concept and points rather to the operative technique, and mainly to the postoperative treatment as being responsible.

If the operation must be supplemented by external fixation, it will undoubtedly influence the result of the healing disadvantageously. Most of our problems arose in connection with ensuring this condition and are not yet solved in many respects even today.

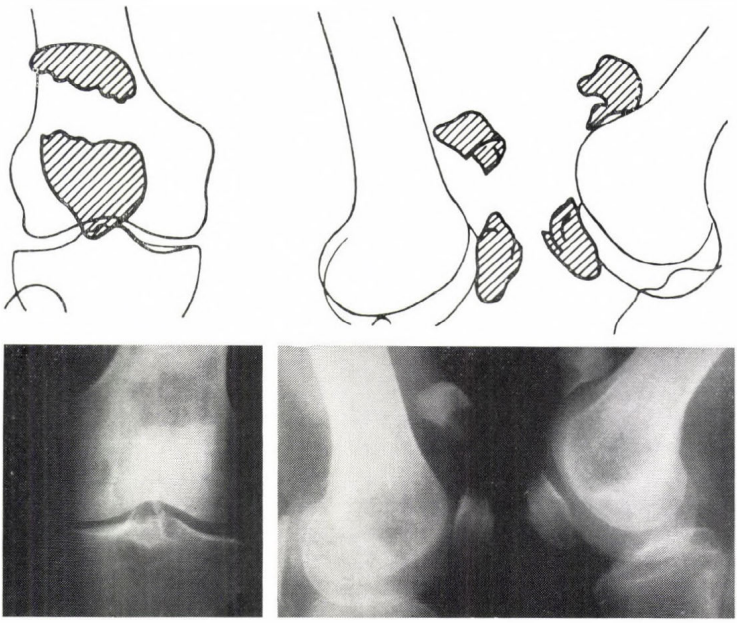


FIG. 1. B. G. 88-year-old man. Trauma (15. 3. 1965); broad diastasis between the fragments of the right patella increasing if the knee is bent; sign of total rupture of the extensor apparatus

The view of the necessity of plaster fixation has become so deeply rooted in the general practice that it is exceedingly difficult to make any headway against it.

It is necessary to mention that one of the fundamental conditions of the treatment is to block the sensation of pain. This problem may be considered to be solved by the administration of Scutamil—C.

Injuries of the associated soft parts: the wounds—interstitial haemorrhage—heal well in the functioning extremity and are absorbed quickly. Neurovegetative and circulatory disorders become easily avoidable complications.

The state of health and disease can, in general, be successfully bridged over by the artificial physiological milieu due to the operation and the reduced function of the organism.

In contrast to general practice, functional treatment should not be suspended on account of inflammatory symptoms, purulence or in case of feverish condition.

A similar procedure should be followed in case of blood and plasma accumulations.

It is striking how much the tissue function can help and to what extent it can facilitate the solution of the complications.

It was found necessary to emphasize the foregoing because in general practice the already started functional therapy is brought to a stop when

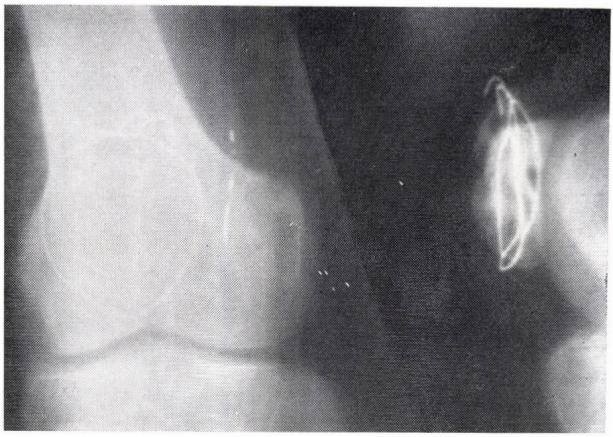


FIG. 2. Control (B. G., 25. 6. 1965); osseous healing with complete function

the symptoms, mentioned above, present themselves. A strict rest is prescribed or else a fixative dressing is applied everywhere.

It is only natural to reduce the extent of the functional stimulus in these cases, and to employ assisted functional treatment similar to that applied in the postoperative period of serious cases.

Taking stock of our 5000 patients with bone injuries treated in the spirit of functional conception since 1958, it may be established that the start was made with timid attempts because not even we dared to trust sufficiently the success of the new treatment, nor were we acquainted with its therapeutic effect. Time, and chiefly the patients themselves, convinced us of the advantages of the novel treatment. Of the methods concerning the treatment of closed fractures, we partly follow Böhler's technique and partly Pap's method of active movement therapy. Of the operative procedures we employ those which make postoperative external fixations unnecessary.

The illustrations are selected from such operated segments which, in practice, cause the surgeon most trouble and are responsible for most failures (knee, elbow, leg, shoulder).

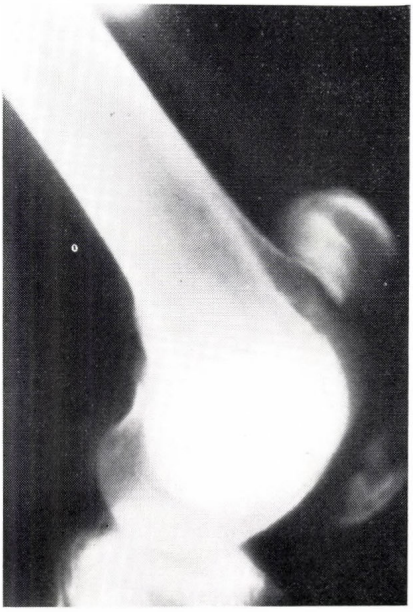


FIG. 3. C. M. 45-year-old man. Trauma (10. 2. 1964); fracture of the left patella with great dislocation and total rupture of the extensor apparatus

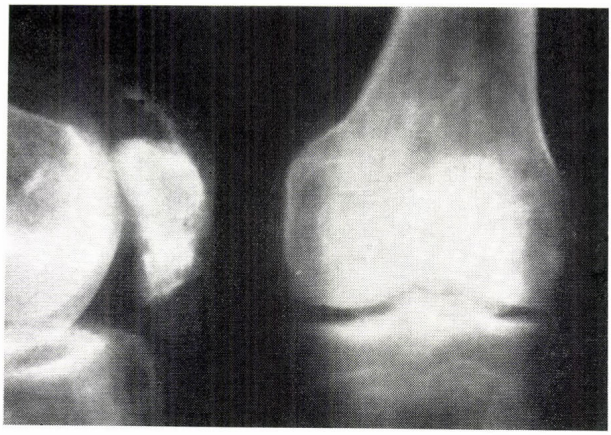


FIG. 4. Control (C. M., 23. 4. 1965.). Re-organization of re-operated patella with complete function. The ramified structure visible in the quadriceps tendon—trace of irritation caused by the wire—did not disturb function

It must have been the lack of knowledge concerning antibiotics and the as yet insufficient sterility of surgical interventions that prevented the propagation of Lister's and Turner's method of intra-osseal patella wire-suture and early functional treatment, although it may be pointed out that the operative and immediate functional treatment of the complete rupture of the extensor apparatus of the knee is a striking proof for the controllability of the tissue differentiation following injury.

A suitable therapy may be decided upon on the basis of anteroposterior and lateral X-ray pictures made of the extended knee and of the lateral and tangent X-ray pictures of the flexed knee.

In the case of milder distraction, the treatment is conservative.

Operative intervention is resorted to only if the extensor apparatus is essentially injured — this is indicated by the distraction of the fragments of the flexed knee.

An intra-osseal U-wire suture and cerclage is carried out from a transverse section. The disrupted ligaments are united with

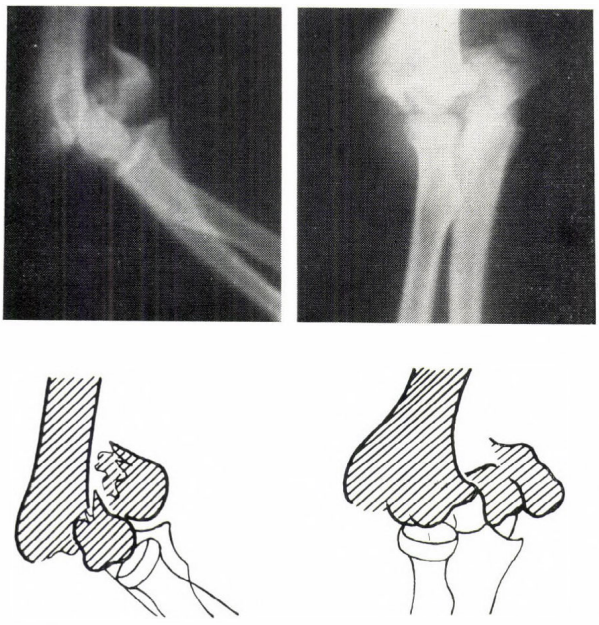


FIG. 5. S. I. 17-year-old man. Trauma (30. 12. 1962); posterolateral sprain of the elbow joint with a fragmentary fracture of the epiphysis of the humerus

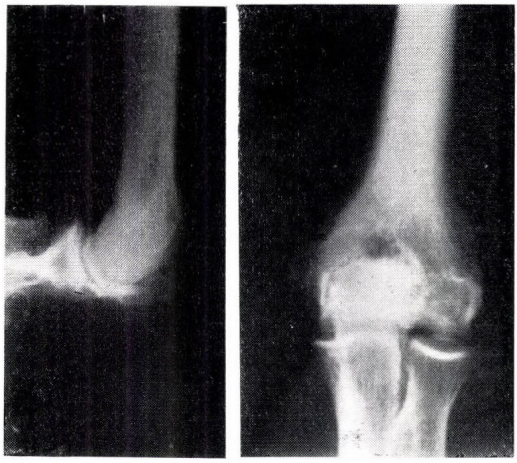


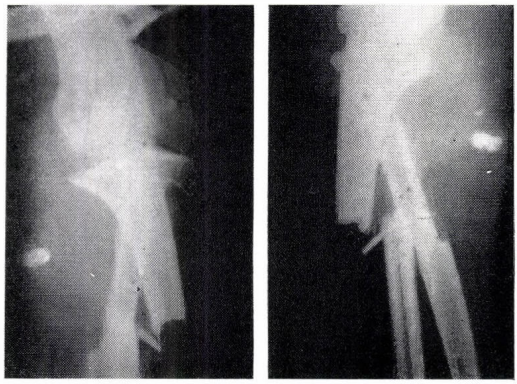
FIG. 6. Control (S. I., 21. 2. 1963). After removal of fixing wires, good position and good function

situated stitches. A compression dressing of elastic bandage is applied. After awakening, the therapeutic programme is reeled off quickly and uninterruptedly: assisted active gymnastics, then gradual gymnastics without any loading, and then walking with crutches.

From the third week on, walks in water, from the fourth week gradual loading. From 8 to 10 weeks a bony unification with perfect functioning ensues in general.

Twenty-four patella fractures were operated (Figs 1 to 4). In fact, two of these were re-operated because the patient fell and the bone sutures came apart. Patella bipartita developed in one of these patients with complete function, while the other patient healed with perfect results even after the second trauma and the repeated operation.

The elbow joint, a no less critical region, is even more prone to become rigid than the knee. It is an easy territory for various internal syntheses: compressor screws; nails; plates; V—X—△ fixations. Even



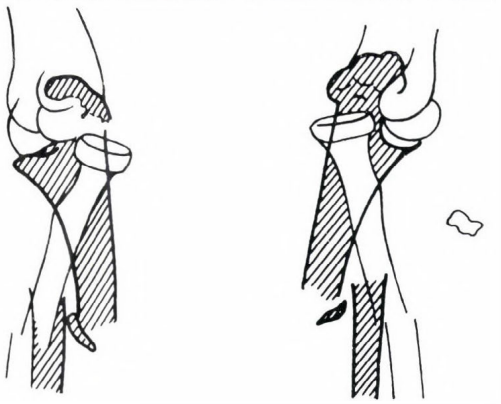


FIG. 7. S. T. 32-year-old man. Trauma (5. 2. 1965); left elbow joint, fore-arm and hand slashed to pieces, sprained and burned. Open fractures of the olecranon, radius, ulna and os naviculare. Median and ulnar nerves and muscles of the fore-arm severely injured

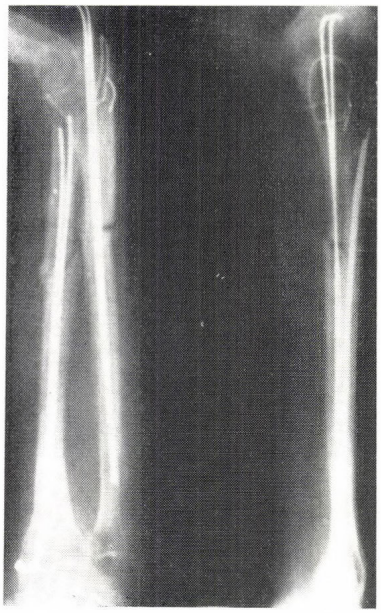
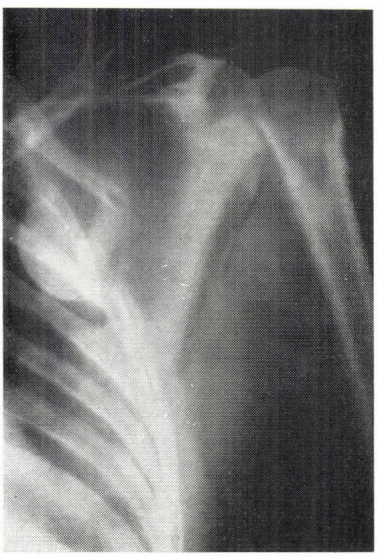


FIG. 8. Control (S. T., 29. 6. 1965). Evident callus formation between the broken bone ends. Functional limits of the elbow joint 40°

in the most complicated cases it seemed sufficient to apply an abductional plaster splint to the extremity temporarily, otherwise it is suspended from the neck by means of a mitella.

As a recent case (Figs 5 and 6) we present the fragmentary fracture of the sprained right elbow of S. I., a 17-year-old man. Campbell penetration followed up by delta fixation, abductional plaster splint for six weeks, at first assisted, later independent unloaded gymnastics daily, use of the extremity. At present he is working as a carpenter with totally restored function.

The left elbow joint of N. I., a 40-year-old joiner, was cut in two by a rotary saw, leaving only a slight ulnar soft spot intact. Osteosynthesis, suture of the nerve (radial—medianus) ligaments, tendons and muscles, a plaster splint applied to



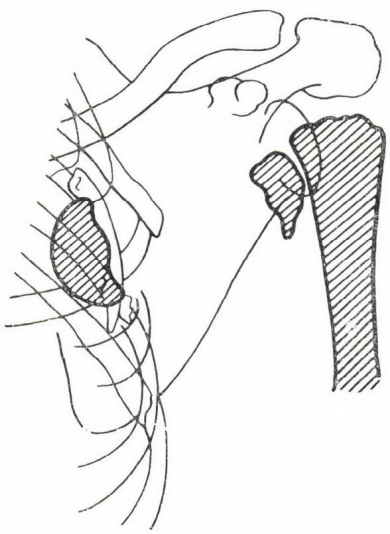


FIG. 9. B. J. 36-year-old man. Trauma (5. 5. 1958); intrathoracic dislocation of the fractured humerus head. The tuberculum majus has broken off. Fractured ribs II to IV

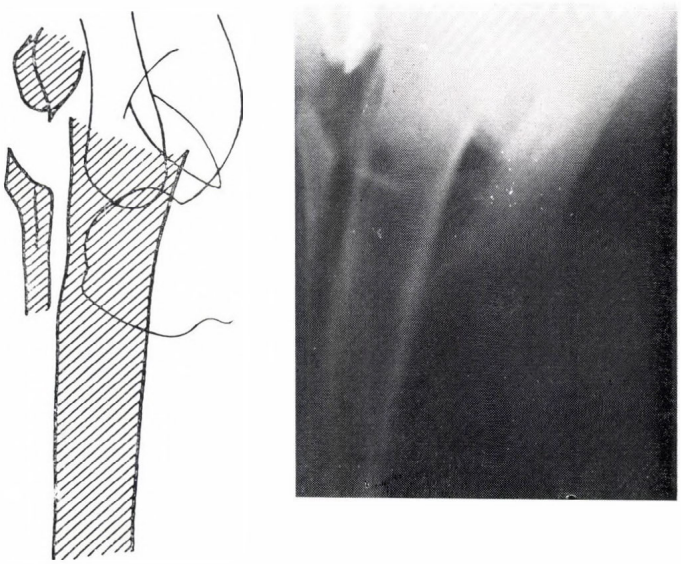


FIG. 10. Axillary X-ray picture of case in Fig. 10. Bone fragments are seen beside the decapitated humerus

the arm, forearm for two weeks. Osseous healing, a 90° motility amplitude in the tenth week. In 18 months the radial and median innervation had become regulated also peripherally.

The hand and left elbow joint of S. T., a 32-year-old technician, was slashed to pieces by the scoop-wheel of a transporter. The left elbow was practically torn in two, the victim suffered an olecranon fracture, a luxatio divergens and fracture of the forearm. His arm was so tightly wedged in that it had to be liberated by means of a welder, and he even suffered third degree burns on his hand. Bone reconstruction, suture of nerves, muscles and tendons, dermatoplasty (skin-grafting), the healing of the bone and soft parts ensued undisturbed in the extremity without any external fixation, the elbow regaining a 70° motility in three months (Figs 7 and 8).

The greater destruction and bone defects can be caused by osteomyelitis. One of the patients who underwent 12 operations and was treated functionally without the slightest fixation, is described.

N. Zs., a 9-year-old girl patient, suffered from sepsis, extensive destruction in the right tibia, contracture in the knee and the ankle. First the duct abscesses were exposed, then drained. A soaking dressing was applied daily, and subwater gymastics were performed. Within a week the patient was able to stand up with the help of crutches.

Under the influence of the functional stimulus, the bone process, apart from propagating destruction, develops a protecting callus sheath everywhere which gradually takes over the static role of the diseased bony part. Complete functional healing ensues. Parents are averse to having the thin sequester removed.

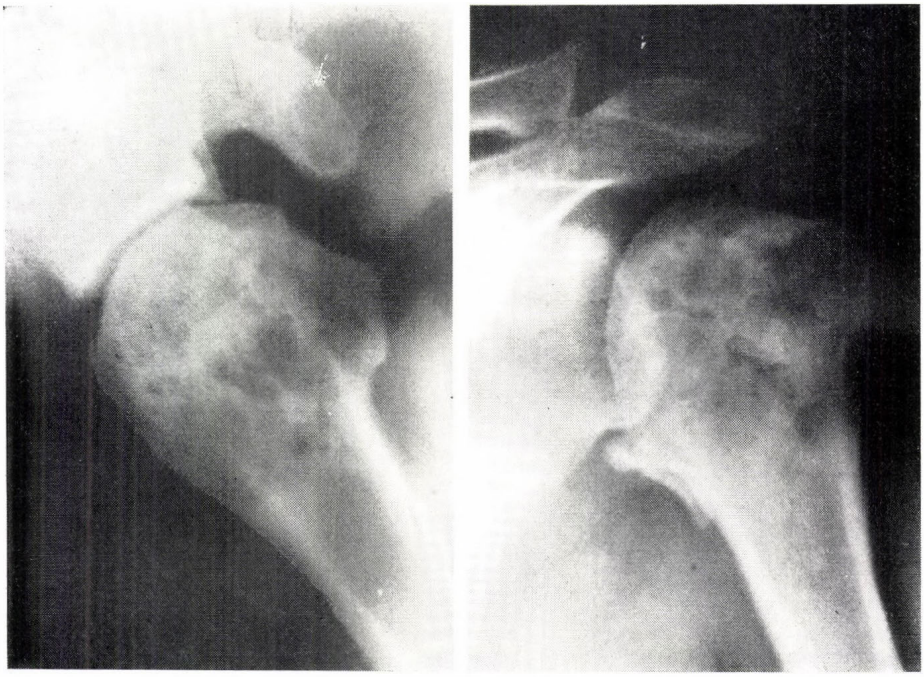


FIG. 11. Control (B. J., 10. 5. 1962). Normal articular gap; the replanted humerus head shows in anteroposterior direction an elliptic configuration (a) and in axillar direction a normal but unequally shadowed picture (b). The implanted rib has been incorporated. No signs of arthrosis. Function: 3/4 of the normal

The luxated fraction of the humerus head is obviously associated with considerable injury of the large soft parts. The closed repositional procedure is often unsuccessful.

Sz. E., a 50-year-old man, suffered a right-sided luxative fracture of the humerus head and an olecranon fracture on the same side simultaneously. The olecranon fracture was united by a U-wire suture, the exposure was accomplished by means of Cubbins penetration, the head was lifted out of the axilla and fixed to the end of the humerus stem with two Kirschner wires bent to the shape of a shepherd's crook, the tubercles were fitted into their places and kept there by two situated stitches. A week later the patient received a Desault dressing (we omit this nowadays), then functional therapy was applied. The healing result was good, the patient has resumed his original occupation as a mechanic.

B. J., a 36-year-old chief mechanic (Figs 9 to 11), fell into the Hortobágy canal with his motor-cycle and suffered a left-lateral intrathoracic dislocation. The fractured humerus head which had slipped into the diaphragm of the heart-angle was removed by thoracotomy, after which a draining osteosynthesis was performed between the re-implanted head and the humerus

stem with a rib segment. The patient wore an abductional plaster splint for ten weeks, performing meanwhile the prescribed gymnastics. The reimplanted head, though somewhat deformed, was incorporated, its articular surfaces are intact, and the function is almost perfect.

SUMMARY

The reciprocal connection of form and function demands integrity, continuity and adequateness of the functional stimulus in order to maintain a state of equilibrium, in other words, a state of health.

In case of injury, a change of form is primary, the *functio leasa* is only its result. Not only does the order of the reparation follow from the foregoing, but also the justification of the formal reconstruction, as well as the demand for an adequate and continuous functional stimulus on the part of the injured organ.

In functional therapy, the adequate functional stimulus is methodically employed for the purpose of directing the regenerating process.

The prominent significance and primary role of function as a therapeutic treatment superior to all others follows as a reasonable deduction of the foregoing.

The cause and effect relations of the above concept are quite evident and can readily be followed in the clinical examples reported as regards the healing of operated and functionally and continuously treated bone fractures.

Symp. Biol. Hung., 7, pp. 227—240 (1967)

EXPERIMENTAL DATA ON BONE INDUCTION AND BONE ANTIGENICITY

by

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This communication deals with two different problems which are being studied at present in the Department of Histology and Embryology, Academy of Medicine in Warsaw. These two problems will be discussed separately, though they have common points, such as general problems of tissue transplantation and tissue incompatibility connected with it.

OSTEOGENESIS INDUCED BY THE BIOLOGICAL INDUCTOR OF THE URINARY TRACT MUCOSA

Osteogenesis induced by the urinary bladder mucosa was described for the first time in detail by Huggins in 1931. This phenomenon is particularly interesting theoretically because it is a manifestation of experimental metaplasia probably based on a chemical mechanism of the same type as that observed in embryogenesis. On the other hand, in plastic and reconstructive surgery this phenomenon seems to indicate the possibility of obtaining a biological inductor of osteogenesis which might be employed in cases of delayed healing of fractures and pseudoarthroses. With this aim in view many workers (Copher 1938, Eskelund and Plum 1950) carried out experimental work, and tried to apply this method in human beings (Wysockaja 1952).

The classic experiment consists of grafting a fragment of autogenous urinary bladder mucosa onto a fascial surface. As a result, the epithelium covering the surface of transplanted fragment proliferates spreading over the connective tissue of the host and lining the cleft between graft and its bed. In this way a cyst is formed completely lined with epithelium (Zaleski 1960). Foci and bone tissue appear in the recipient part of the cyst wall. This experiment also succeeds when allogenic grafts are used, but only if the time necessary for bone tissue induction is shorter than the period required for graft rejection, because of tissue incompatibility. This refers to guinea-pigs, cats and Syrian hamsters.

All attempts of bone induction failed by xenogenic grafts (Zaleski et al. 1963). Various papers report the phenomenon of induction of osteogenesis in various animals, such as the cat (Johnson and McMinn 1955, 1956), the rat (Blumel and Piza 1958), the guinea-pig (Friedenstein 1956), Syrian

hamster (Zaleski 1961), the mouse (Zaleski and Moskalewski 1963) and also in man (Welcker 1950).

In spite of numerous investigations undertaken to elucidate the mechanism of bone induction, many points remain obscure and opinions are controversial. Table I presents the problems investigated in our department.

TABLE I

Subject of investigation	Method
Bone induction in various animal species	Urinary bladder grafts
Dependence of bone induction on age of donor and host	Urinary bladder grafts from foetus to adult and from adult to new-born
Some histochemical traits of transitional epithelium	PAS reaction Alkaline phosphatase reaction
Bone induction by isolated and cultured *in vitro* epithelial cells	Grafts of cell cultures and cell suspensions
Bone induction without direct contact of grafted epithelium and host connective tissue	Grafting of epithelium in diffusion chambers Experiments with surgical 'small urinary bladder'
Morphology and development of induced bone	Roentgenography Intravital staining Labelling with isotopes

All autogenous grafts of vesical mucosa in dogs induced bone formation in their vicinity. However, allogenic transplants in these animals perished owing to tissue incompatibility destroying the graft without producing osteogenesis (Ostrowski et al. 1957).

In guinea-pigs osteogenesis in the neighbourhood of about 70 to 80% of allogenic grafts has been demonstrated on the tenth day after grafting (Fig. 1).

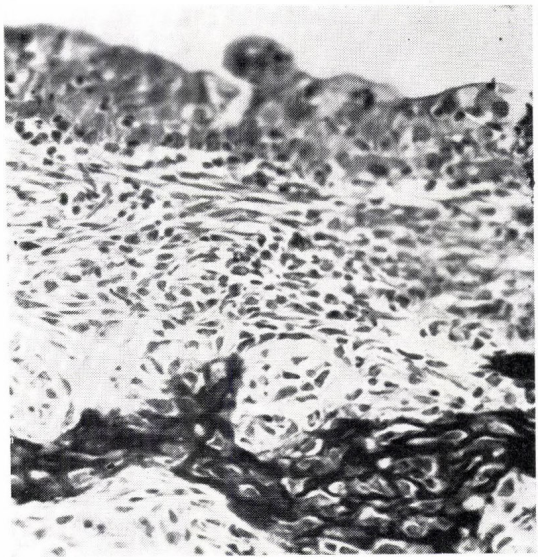
Osteogenesis was also observed around allogenic transplants in hamsters, but it occurred much later and in a smaller percentage of cases than in guinea-pigs (Zaleski 1961).

The bone tissue around autogenous grafts was demonstrated very rarely in mice (Zaleski and Moskalewski 1963).

When vesical epithelium from 30-day-old guinea-pig foetus was used as material for transplants, a cyst lined with epithelium as well as bone tissue within its walls formed just as frequentely as in grafting urinary bladder epithelium from mature individuals (Zaleski 1962a). It was also demonstrated that in 2 to 4-day-old guinea-pigs, bone tissue can be induced by allogenic grafts of urinary bladder epithelium.

The occurrence and distribution of PAS-positive substances and alkaline phosphatase were studied in the transitional epithelium of guinea-pig and

FIG. 1. Bone tissue in a 10-day graft of allogenic urinary bladder of guinea-pig. PAS reaction; Haematoxylin



man, both in mature individuals and foetuses as well as in transitional epithelium of Syrian hamsters (Zaleski 1961, Zaleski et al. 1963). The histochemistry of transitional epithelium was also studied in tissue cultures (Moskalewski 1960).

Alkaline phosphatase was found in the basal layers of transitional epithelium in hamsters and guinea-pigs, whereas in human beings this enzyme was present only in foetuses aged 12 to 14 weeks. In this case it was exclusively in the superficial cell layers. Alkaline phosphatase was also present in some guinea-pig cells cultured *in vitro*. It could not be detected in the epithelium of guinea-pig foetuses and in adult human beings and in foetuses after the 16th week of gestation.

Glycogen was mainly found in the middle and superficial layers of transitional epithelium in hamsters and guinea-pigs. Considerable quantities of glycogen occurred in all layers of foetal epithelium both in guinea-pigs and in human beings. In adult humans no glycogen was found, perhaps because the material was taken from individuals at operation who have fasted for some 12 hours or so.

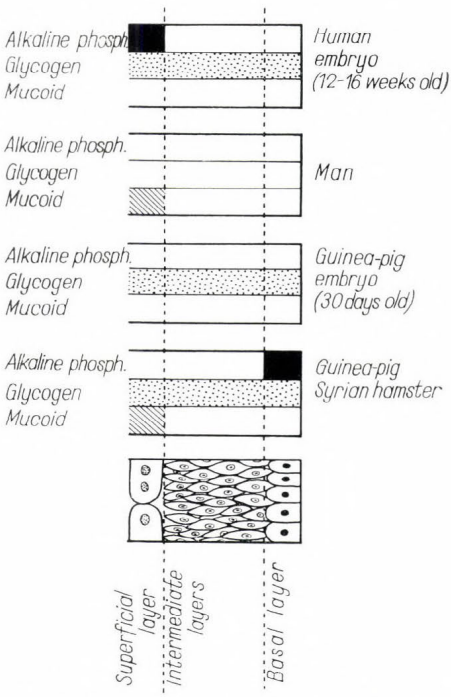


FIG. 2. Diagrammatic representation of results obtained from histochemical investigations of transitional epithelium

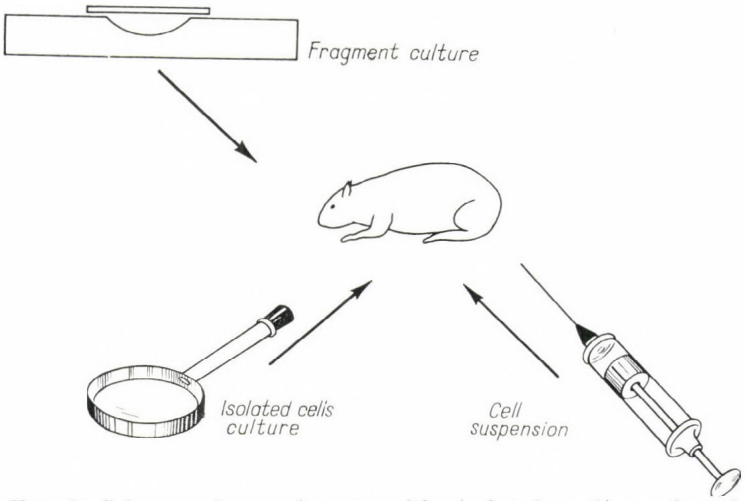


FIG. 3. Scheme of experiments with isolated and/or cultured *in vitro* transitional epithelium of guinea-pig

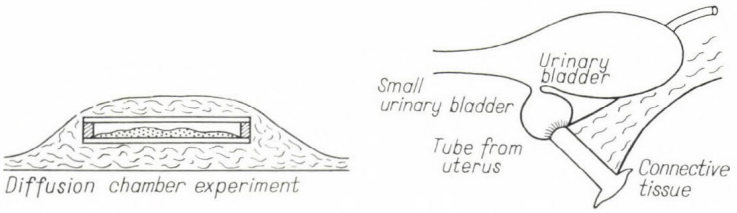


FIG. 4. Scheme of experiments in which transitional epithelium was separated from direct contact with connective tissue.
Left: Experiments with diffusion chambers; Right: artificial 'small urinary bladder'.

Mucoid is found in the surface layers of the epithelium of guinea-pig, hamster and human beings. In human foetuses it appeared only in later stage of foetal life. The same substances (glycogen and mucoid) were demonstrated in cultured epithelium. Figure 2 presents the results obtained from histochemical investigations.

To establish inductive potency of transitional epithelium cells under various conditions, three experiments were carried out as seen in Fig. 3 (Ostrowski and Moskalewski 1959, Ostrowski et al. 1961, Moskalewski 1963).

The epithelium with a fragment of vesical mucosa of guinea-pig was cultured by the double-slide method of Maximow for 3, 5, 8 and 14 days. Transplants were then taken from these cultures. Induction of osteogenesis was observed even 14 days after culturing.

In the second experiment transitional epithelium cells separated from other tissues of urinary bladder by trypsin were cultured. Ten-day-old

cultures were transplanted into connective tissue of allogenic recipients and osteogenesis was achieved. After grafting cultured epithelium underwent a secondary differentiation, although it sometimes exhibited greater vacuolization than epithelium transplanted directly without cultivation *in vitro*. The transitional epithelium cells isolated by trypsin were injected intramuscularly. After injection epithelium cells formed compact islands, around which regular bone formation was observed. Probably on account of a small diameter of the fragment injected, cysts were found only occasionally. The injected suspension may have contained a certain number of connective-tissue cells. Nevertheless, the probability of contact and interaction between them and epithelial cells after injection to recipient is too small to play an essential role in the induction process.

According to some authors (Friedenstein 1960) the bone induction is mediated by some chemical compounds secreted by epithelial cells. To check this, attempts of induction avoiding direct contact of grafted epithelium and host connective tissue have been made (Ostrowski et al. 1961; Fig. 4). Fragments of vesical mucosa were placed in Algire chambers coated with a filter 150 μ thick with pore diameter sufficiently small to retain cells inside the chamber. In the connective tissue surrounding of the chambers no induction of osteogenesis could be obtained, although the epithelium in the chamber survived. In similar experiment Friedenstein (1962) obtained bone formation around the diffusion chambers. In another experiment with female dogs, a 'small bladder' was formed, operatively isolated from the other parts of the urinary tract. The excretions from this artificial small bladder flowed into the connective tissue through a tube formed from part of the horn of uterus. The neighbourhood of the outlet of the tube revealed no bone formation.

According to histological, histochemical and radiological observations and by marking the forming bone tissue with tetracycline, alizarin red S, ^{32}P-phosphate, ^{35}S-sulphate and ^{3}H-glycine, its has been established that up to about the 30th day after grafting of allogenic urinary bladder tissue the induced bone exhibits typical features of young woven bone. The subsequent period showed a picture of intensive bone-tissue resorption which was manifested by the presence of numerous osteoclasts and by reduced radio-opacity.

In the final stage, about 3 to 4 months after transitional epithelium grafting, the formation of lamellar bone tissue was revealed showing signs of both, resorption and of deposition of new bone substance. Evidence of the latter is given by autoradiographs showing ^{3}H-glycine incorporation (Fig. 5). At this time transitional epithelium was no longer present (Zaleski 1962a). Moreover, at this time typical bone marrow was found in the vicinity of bone tissue (Fig. 6). Cytological analysis did not reveal any differences between induced and normal bone marrow (Czerski and Zaleski 1962, Ostrowski et al. 1962).

Table II contains the chief features of induced bone during its development.

When comparing the mineral metabolism of the induced bone, that of callus, compact bone and dentine, the first was found to have a 30 times

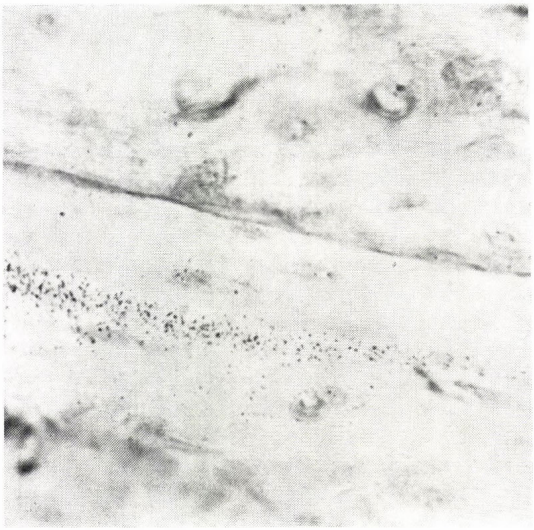


FIG. 5. Autoradiograph of induced bone tissue 70 days after bladder tissue grafting. Note silver grains due to incorporated ^{3}H-glycine along the margin of bone tissue

higher incorporation rate as regards ^{32}P-phosphate (Ostrowski and Wilczynski 1957).

The results of the experiments performed lead to the following conclusions.

1. The phenomenon of osteogenesis induction appears to be associated with the function of living epithelium irrespective of whether it originates from an adult individual or a foetus and whether it is cultured *in vitro* or isolated by trypsin. Cyst formation is not absolutely necessary for bone induction.

2. No relationship could be established between the bone inducing property of transitional epithelium and some of the investigated histochemical substances contained in the epithelial cells.

3. The phenomenon of bone induction by autogenous grafts of transitional epithelium has been observed in many animal species. It may also be revealed in allogenic grafts in those species where destruction of grafted epithelium, depending on tissue incompatibility, does not take place too early.

4. Bone-tissue formation could not be induced if the transitional epithelium was not in direct contact with the connective tissue of the recipient.

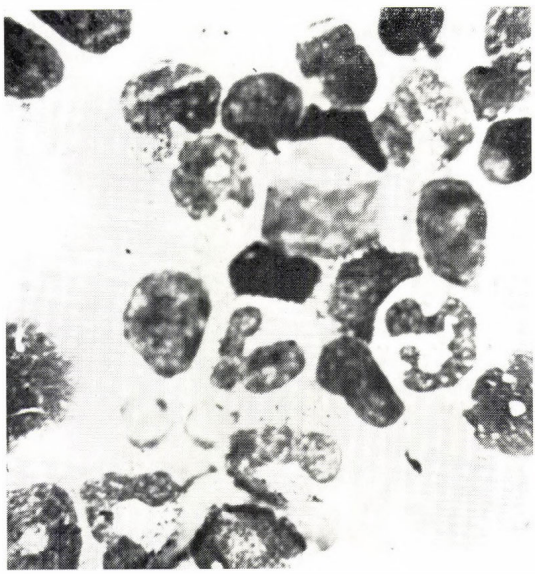


FIG. 6. Smear of induced bone marrow. Its normal appearance draws attention. May — Grunwald—Giemsa

TABLE II

	Days after grafting			
	5–8	10–25	30–50	70–100
Tissue	Connective	Woven bone	Resorbed woven bone	Lamellar bone
PAS reaction	Negative	All bone matrix +	All bone matrix +	Rare positive areas
Alizarin red S	Negative	All bone stained	White superficial layer on stained bone	Rare stained areas
Tetracycline	Negative	Peripheral trabeculae strongly +	—	Superficial layer strongly +
^{35}S-Sulphate	Diffusely distributed grains	Numerous grains over peripheral trabeculae	—	Numerous grains over bone connective-tissue border
^{3}H-Glycine	—	Linearly distributed grains over trabeculae	—	Linearly distributed grains over marginal and perivascular layers of bone

5. Three main phases may be distinguished in the development of induced bone tissue: (a) connective tissue formation, (b) induction conditioned by the transitional epithelium and (c) autoinduction. The last probably depends on the osteogenic action of substances formed in the course of bone-tissue resorption.

6. Bone tissue in the autoinduction phase shows vitality and metabolic activity, in spite of the absence of transitional epithelium.

7. In the last phase induced bone is accompanied by active haemopoietic tissue which does not differ from the normal bone marrow of the host cytologically.

REGIONAL LYMPH NODE REACTION FOR THE ASSESSMENT OF BONE TISSUE ANTIGENICITY

Certain characteristic morphological and cytological changes occurring in the regional lymph node under the influence of tissue graft were described for the first time by Gallone et al. in 1952. They found that such lymph

node loses its typical nodular structure, and then morphologically immature cells appear in greater number than normally. These cells will be termed blastic cells.

Several authors supported and developed these observations (Egdahl et al. 1958, Scothorne and McGregor 1955). Recently, attempts were made to elaborate an objective and quantitative method for the assessment of cytological changes (André et al. 1962, Burwell and Gowland 1960). Unfortunately, none of these methods is very precise and, therefore, our aim was to elaborate a simple and standardized method to assess the changes in percentage of blastic cells under various experimental conditions.

Rabbits were used in all experiments. Small fragments of allogenic or xenogenic cancellous or compact bone tissue were grafted in the vicinity of auricular lymph node. After a given time this node was removed and a cell suspension was prepared. In the smears from these suspensions the percentage of blastic cells and plasmacytes was estimated using the Woolf method (1950) for cell counting.

It was established that under the influence of allogenic and xenogenic tissue graft there is statistically significant rise of blastic cell percentage (Zaleski et al. 1964; Fig. 7). The percentage of plasmacytes does not differ from the normal value. Additionally, it was found that intensity of blastic reaction evoked by allogenic and xenogenic grafts is the same, but the duration varies greatly. The percentage of blastic cells in the lymph node draining the allogenic graft area falls to the normal level after two weeks, while in xenogenic graft it drops after three months. This method of assessment of blastic reaction is simple and gives average results with relatively smaller standard deviations than the methods used by other authors.

Blastic cells have typical morphological features, i.e. strongly basophilic cytoplasm, large pale nucleus with very delicate chromatin structure and 1 to 3 distinct nucleoli (Fig. 8). Among them three types of cells can be distinguished. They correspond to lymphoblast, plasmablast and large lymphoid cells. Attempts of objective differentiation of these cell types failed because histochemically they do not differ. Therefore, they were classified in a common group of blastic cells (Rymaszewska et al. 1963).

A practical application of this method was carried out using banked bone tissue. As suggested, some procedures used for preservation of tissues lead to decreased antigenicity of preserved tissue. On the basis of blastic reaction evoked by fresh and preserved xenogenic bone tissue, it is possible to state that there is no change in the percentage of blastic cells appearing after these two types of grafts (Zaleski et al.1964). Further experiments are needed to determine whether changes in the antigenicity of tissue ever occur after conservation procedure.

Recently it was established (Zaleski et al.) that the method is applicable for the assessment of cytological reaction of lymph node draining the injection site of soluble antigen HSA. In this case, after an increase of blastic cells there is a rise of plasmacyte percentage. It indicates the pathway of differentiation of blastic cells to plasmacytes. On the other hand, immunofluorescent studies by Coon's method revealed the presence of cells containing specific antibodies, anti-HSA. The blastic reaction evoked by

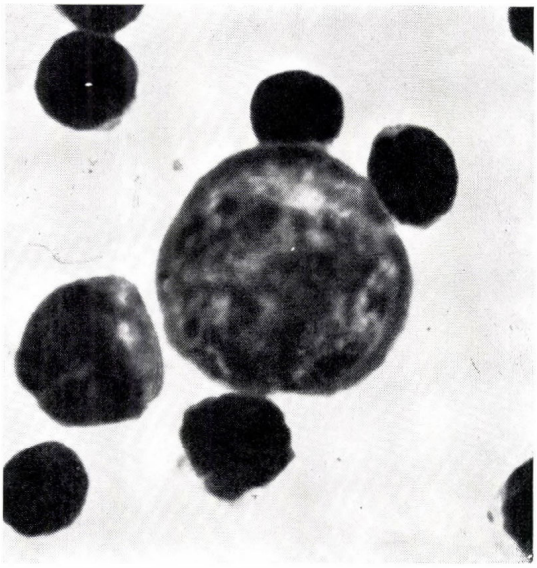


FIG. 7. Typical blastic cell. Large cell with delicate structure of nucleus and wide basophilic ring of cytoplasm. May—Grunwald—Giemsa

HSA is influenced by the dose of antigen and is smaller after an extremely large dose. Further studies have shown that in specifically tolerant animals there is only a trace of blastic reaction without any changes in plasmacyte percentage (Fig. 9). At the same time, in this case we found no cells containing specific antibodies.

Purely morphological observations do not allow an estimation of the role of blastic reaction and blastic cell itself in the process of graft rejection. For this reason, autoradiographic studies using tritiated glycine were performed to observe the intensity of protein synthesis by lymph-node cells (Zaleski et al. 1965).

It was established that there is no difference in the protein synthesis by single cell in normal or graft stimulated lymph nodes. However, because of a rise in the percentage of blastic cells after grafting, total protein synthesis seems to be greater in the stimulated nodes (Fig. 10).

Ehrich and Harris (1942) found that after local introduction of bacterial or protein antigens there is an increase in the number of cells leaving the regional lymph node with lymph. At the same time, specific antibodies with increasing titre appear in the lymph.

It appeared worth while to study whether any changes in the morphology and protein content of lymph occur after tissue graft. Such investigations would give us new possibilities for the study of two important points: (a) the fate of immunologically competent cells appearing in the regional lymph node, and (b) the role and presence of humoral

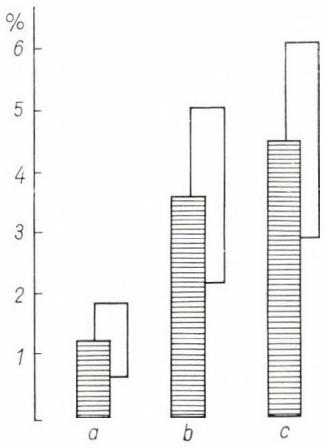


FIG. 8. Percentage changes of blastic cells in normal lymph node (a) and in lymph node after allogenic (b) and xenogenic (c) grafts. The striated columns represent average values and the white columns represent standard deviations

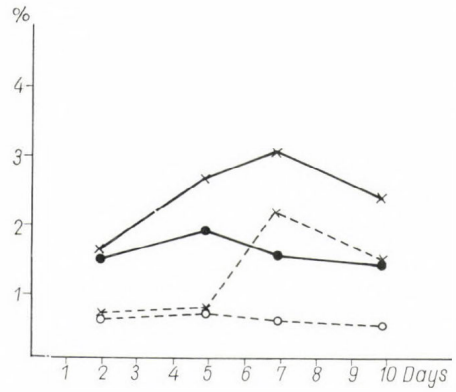


FIG. 9. Changes of the percentage of blastic cells (————) and plasmacytes (– – – – – –) in lymph nodes draining the injection site of HSA in normal (X) and tolerant (•) rabbits

antibodies in the tissue graft rejection. With this end in view, an attempt was made to drain the lymphatic efferent vessel of node draining the tissue graft area. On studying the lymph leaving the regional lymph node stimulated previously both by allogenic (Włodarski et al. 1964) and xenogenic grafts (Rymaszewska et al. 1965), it was established that there is an increase in the number of cells flowing out from regional lymph node and, simultaneously, the percentage of blastic cells in the lymph rises 3 to 4 times. At the same time, the total protein content and electrophoretic fractions in the drained lymph do not change. Moreover, this lymph does not exert any cytotoxic effect on the target cells of the graft donor. These results seem to support the hypothesis of the cellular character of tissue incompatibility. It is impossible to say whether a single graft is able to cause antibody formation detectable by the methods employed.

To check this problem experiments with blastic reaction evoked by two succesive grafts were performed (Rymaszewska et al., Zaleski et al.). The fluctuation of reactivity of regional lymph node was established by using two successive allogenic grafts. Depending on the time interval between the two grafts, different response of regional lymph node was observed (Fig. 11).

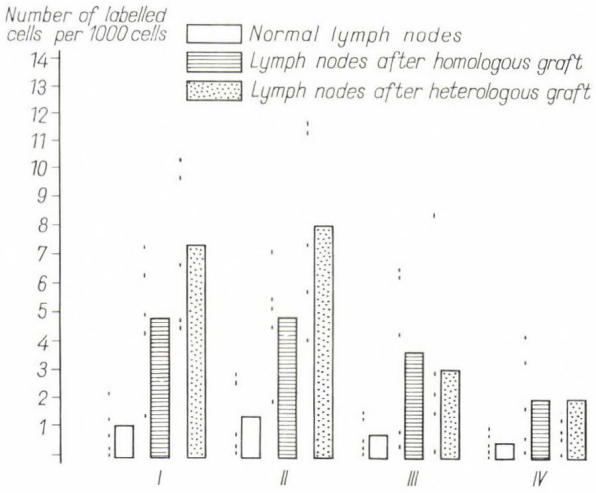


FIG. 10. Absolute number of labelled blastic cells divided into 4 groups depending on labelling intensity. Each column represents the average value obtained from 5 results marked as black dots beside the relevant columns

In the third and twelfth week after the first graft, the second set graft gives an increase of blastic cell and plasmacyte percent-

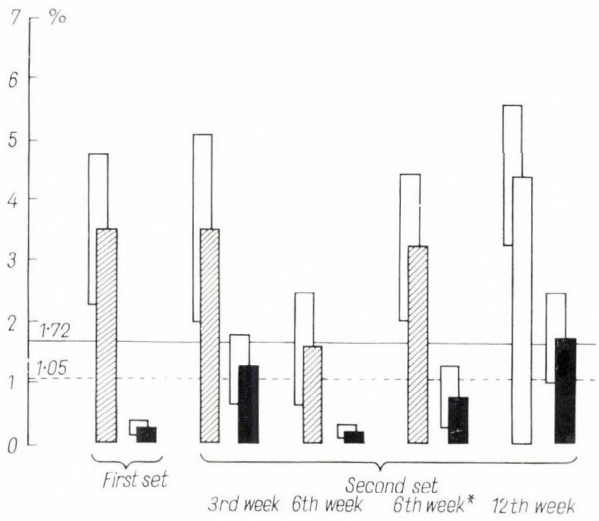


FIG. 11. Percentage of blastic cells (striated columns) and plasmacytes (black columns) in the regional lymph nodes 5 days after the first and second set grafts. White columns represent standard deviations. The continuous and dotted lines indicate the upper limits of normal values of blastic and plasma cells, respectively

age, while in the sixth week the second set graft does not evoke any changes in the cytology of lymph node. The mechanism of these changes is obscure and further experiments are necessary to classify it.

It is known that 'areactivity' observed in the sixth week is species-specific, i.e. allogenic grafts from the same or different donors do not evoke any reaction, but at the same time xenogenic graft does. Moreover, this areactivity concerns only the previously stimulated lymph node, while another node of the same recipient gives typical reaction. It is possible that species-specificity of secondary reaction depends on common antigenic determinants present in a given population of animals but cross-reaction with unrelated antigens cannot be excluded.

Considering the mechanism of secondary reaction, it was found that the percentage of blastic cells increases much earlier than during reaction evoked by the first set graft. Moreover, decrease in their percentage is accompanied by a rise in plasmacyte percentage (Fig. 12). It indicates that there is

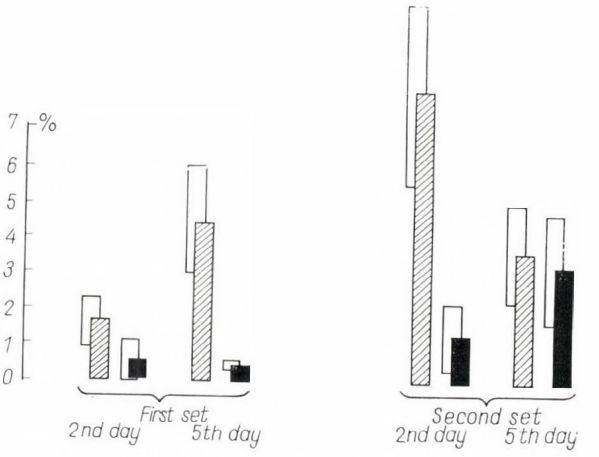


FIG. 12. Changes of blastic (striated columns) and plasma cells (black columns) percentage on the 2nd and 5th day after the first and second set xenogenic grafts. White columns represent standard deviations

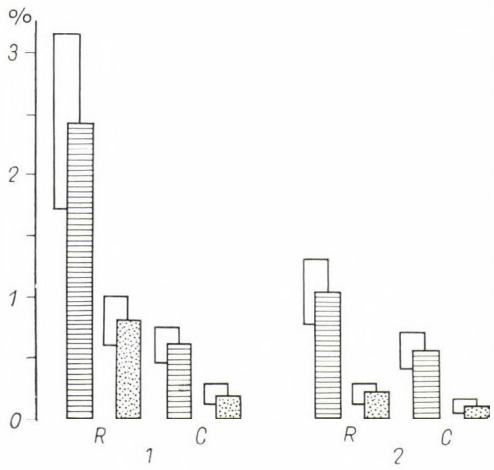


FIG. 13. Percentage of blastic cells (striated columns) and mitoses (dotted columns) in the regional lymph nodes (R) draining the skin graft across H2 barrier (1) and across H3 barrier (2). The percentage of blastic cells and mitoses in the respective contralateral lymph nodes (C) is also given. White columns represent standard deviations

differentiation of blastic cells into plasmacytes which was not observed during reaction evoked by the first set graft.

Increase of the percentage of plasmacytes suggests increased γ-globulin production and, therefore, the presence of humoral antibodies. This leads to studies by electrophoresis and immunodiffusion as well as by a cytotoxic test of the lymph leaving the lymph node draining the area of the second set graft. These studies are in progress.

Finally, the blastic reaction in the regional lymph nodes draining the skin grafts in mice was investigated (Zaleski and Wiklický). In comparison with rabbits, this blastic reaction in mice develops later and was observed on the 11th day after grafting. It is noteworthy that the average survival time of skin graft in both experimental animals is approximately ten days. As seen, there is no relationship between survival time of graft and blastic reaction, and it is wortwhile to study the correlation between these two phenomena.

It was also ascertained that the intensity of blastic reaction at the given time after grafting is dependent on the antigenic differences between donor and recipient. Eleven days after skin grafting across H2 barrier there is higher reaction than at the same time following skin grafting across H3 barrier (Fig. 13). A very interesting, but at the same time obscure, relationship between blastic reaction in the regional lymph node and cytological changes in the thymus were observed. After skin grafting in mice there is a decrease of blastic cells in the thymus. The greater the genetic disparity between donor and recipient, the smaller the decrease of blastic cells in the thymus. Only a very speculative explanation for this fact can be offered at present.

To summarize the results obtained the following can be stated.

1. Both tissue antigens and soluble HSA, evoke an increase of percentage of cells, termed blastic, in the regional lymph node.

2. These blastic cells have some typical properties: they divide, they differentiate into plasmacytes and they synthesize protein. The differentiation takes place during the first immunization with soluble antigen and during the second set graft.

3. Blastic reaction in the lymph node is accompanied by an increase of the total number of cells leaving this node. At the same time there are no changes in the total protein content and their electrophoretic fractions in the lymph.

4. The second set graft, depending on the time interval between two successive grafts, evokes the reaction with a rapid and high rise of blastic cell percentage followed by an increase of plasmacytes. This secondary reaction is relatively unspecific and appears under the influence of two rather unrelated antigenic stimuli.

5. Cells appearing under the influence of soluble antigen contain specific antibodies which are absent in the lymph node of tolerant animals. In these animals the blastic reaction is almost negligible.

6. So far there are no data concerning the relationship between the survival time of the graft and the blastic reaction evoked by this graft.

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Symp. Biol. Hung., 7, pp. 241—250 (1967)

BONE REGENERATION UNDER THE INFLUENCE OF EGG-SHELL GYPSUM AND METACRYLATES

by

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The use of autoplastic, homoplastic and heteroplastic bone material in surgery goes back to the first half of the 19th century (Heine et al. 1964). After the recent discovery of materials not hostile to tissue, alloplastics came more to the foreground in experiments and in clinical use, especially in orthopaedics.

It is indisputably due to Krompecher's basic investigations on the biological use of egg-shells (1958) that a new impulse has been given to further experiments with alloplastic materials. The importance of the egg-shell application for callus production is evident from the investigations of Lelkes and Mészáros (1957). These authors were able to establish that experimental animals which have been fed with egg-shells showed a quicker and better healing of fractures of bones in comparison with control animals. On the basis of this statement, Tarsoly (1963), Tarsoly and Tomory (1963) applied a mixture of tyndallized egg-shell powder and sterilized gypsum in smaller and larger drill defects of bones of dogs (tibia, femur). This reaction of gypsum on bones is known, e.g. from Fründ's investigations (1954). The histological investigations showed that this mixture fastens bone regeneration in comparison with non-plombed control borings. Bornemisza and Bakó (1958) obtained similar effects by using polymethylmetacrylate shavings. Metacrylates are known to be tolerated well (protheses in stomatology, endoprotheses in surgery) due to their contact proliferation (Heinze 1955, Matzen 1955, Seyfarth 1955). By following Krompecher's basic concepts and suggestions, we compared egg-shell gypsum and plast plombs, the results of which are reported in detail.

MATERIAL AND METHOD

The experimental animals were 10 full-grown rabbits of both sexes, aged 6 to 9 months and weighing 3000 to 4000 g.

Operative technique. Intravenous narcosis in the ear vein with a 2% hexobarbitalnatrium solution (0·15 g/kg). Skin cut above the medial tibia area, preparation of the tibia; periosteum removed. On either side three drilled holes were made at distances of 1 cm from the proximal to the distal direction, having a diameter of 3·5, 3·0 and 2·5 mm, through the compacta into the marrow cavity (Fig. 1). All three borings of the left

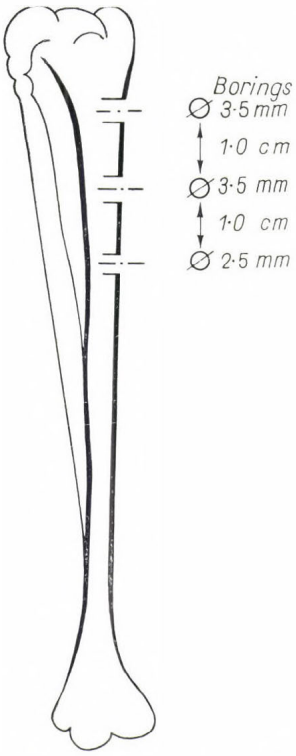


FIG. 1. Arrangement of drillings on rabbit tibia

tibia were plombed; those of the right tibia were left empty for control. *Fillings:*

1. in 5 animals a mixture of tyndallized eggshell powder and sterilized gypsum were applied in a proportion of 1 : 1 and with a grain size of $< 60\ \mu$. A paste was prepared with normal saline solution to which 20,000 U of penicillin was added;
2. in 5 animals the filling was made with splintered polymerisate-piacryl DF (2), of a grain size of $< 60\ \mu$, previously sterilized in a water bath.

Since the periosteum was too thin and tender, the defects were covered with fasciae of the musculus tibialis anterior and flexor digitorum pedis which were united with catgut above the defect. Skin suture was made with silk. Healing took place in all cases *per primam intentionem.* The animals were killed after 2, 3, 4, 6 and 12 weeks. Having been fixed in 10% or 4% formaldehyde, the bones were decalcified in 5% nitric acid. Paraffine embedding, serial sections of 12 to 15 μ, and azan staining was made according to Heidenhain.

The histological assessment refers to 14 sections of each group. In our assessment chiefly the spatial and quantitative expansion of the callus and the connective tissue (Figs 2 to 5) were studied according to the following points:

I. Inner callus, i.e. expansion of the endostal callus below the drill point;
II. Marrow callus, i.e. filling of the marrow area by the callus;
III. Connective tissue proliferation in the marrow area;
IV. External callus, i.e. expansion of the periostal callus above the drill point;
V. Apposition callus, i.e. callus outside the drill point;
VI. Connective tissue in the drill canal;
VII. Marrow substance in the drill canal;
VIII. Osteoid tissue in the drill canal;
IX. Density of the osteoid tissue in the drill canal.

The preparation was assessed as follows:

nought = 0	up to 60% = 3
up to 20% = 1	up to 80% = 4
up to 40% = 2	above 80% = 5

The density of the osteoid tissue is also given in the assessment of 0 to 5.

Statistical evaluation. For each of the nine groups the arithmetical means of the 14 assessed sections was calculated, and the corresponding group value of the control drillings was substracted from this. The resulting values were temporally compared in the groups I to IX (egg-shell powder gypsum mixture = E, splintered polymerisate = M).

By a variance analysis we established that the values of a tibia found in the three drill hole sizes showed no statistical differences. For this reason, we deliberately omitted to differentiate as to drill hole sizes by calculating the arithmetical means of the values of the three drill hole sizes. The significant calculation of the values found was made according to the T-test. In Figs 5 and 6 the results of egg-shell gypsum (E) and plast (M) were compared for each group I to IX. Then the significance of the compa-

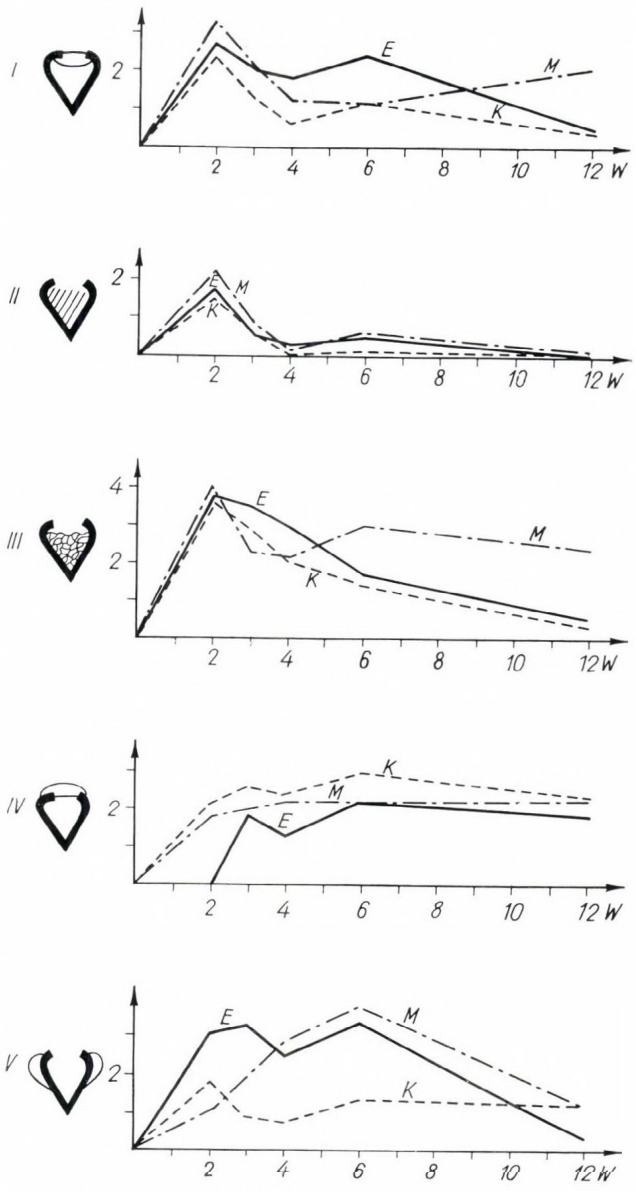


FIG. 2. Diagrams of groups I to V

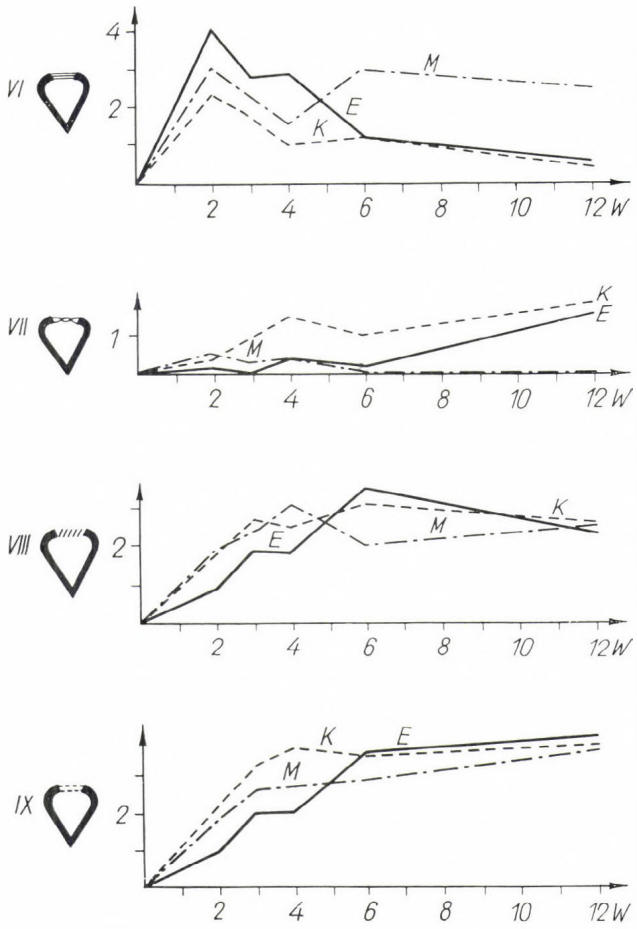


FIG. 3. Diagrams of groups VI to IX

rison values was calculated according to the Rang test and the T-test.

RESULTS

Groups I to IX show the values of the reactions with egg-shell gypsum plombs (E), metacrylate plombs (M) and control drillings (K) which were obtained from the arithmetical mean of the original values of the three drill hole sizes of one tibia.

Group I, inner callus. High initial values in the second week for (M),(E) and (K). Then follows a decrease up to the fourth week. In the sixth week the value of (M) corresponds to that of (K), but (E) is considerably higher. In the twelfth

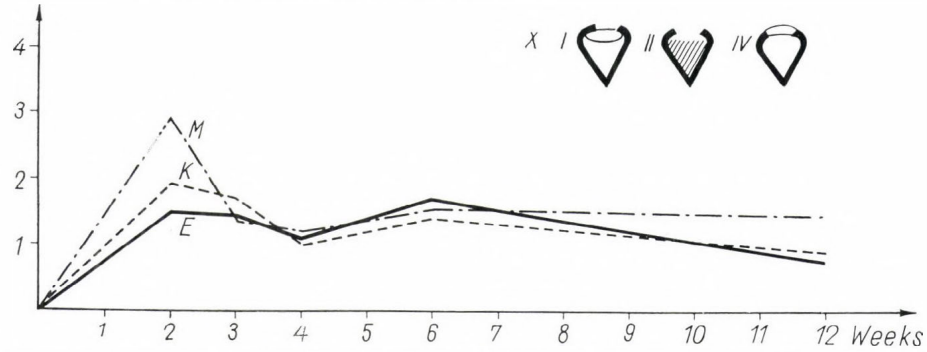


FIG. 4. Diagram of group X. I = inner callus; II = marrow callus; IV = external callus; E = egg-shell gypsum; M = metacrylate; K = control

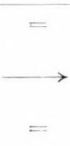
	Inner callus	Marrow cav. call.	Conn. tiss. in. cav.	External callus	Apposit. callus	Filling up of the hole by			
						Conn. tiss.	Marrow	Osteoid tissue	Density of osteoid tiss.
	I	II	III	IV	V	VI	VII	VIII	IX
									
(E) Filling minus Controll (K)									
2 weeks	1·16	1·03	0·33	— 2·..2	— 1·60+	1·95	— 0·36	— 1·58	— 1·24
3 weeks	0·92	0·41	0·59	— 0·04	— 0·26	1·33	— 0·35	— 1·28	— 1·48
4 weeks	1·23	0·26	1·38	— 1·05	— 2·04	1·49	— 0·76	— 0·69	— 1·64‡
6 weeks	0·39	0·23	1·14	— 0·55	— 2·88‡	— 0·43	— 0·12	— 0·07	— 0·01
12 weeks	0·28	0	0·36	— 0·26	— 0·62	0	— 0·40	— 0·24	— 0·36
Tendency									
(M) Filling minus Control (K)									
2 weeks	0·14	— 0·04	0·17	0·12	— 1·04	0·46	— 0·06	— 0·39	— 0·30
3 weeks	0·70	0·05	— 0·52	— 1·14	0·04	0·92	— 1·04	0·09	— 0·30
4 weeks	0·59	0·23	— 0·29	— 0·33	1·18	1·54‡	— 1·54	0·64	0·09
6 weeks	0·19	0·57	1·11	— 0·21	1·14	2·23+	— 0·81	— 0·22	— 0·33
12 weeks	1·55	0·11	0·85‡	— 0·36	— 0·21	2·17‡	— 1·28	— 0·65	— 0·26
Tendency									

FIG. 5. Groups I to IX. Comparison of the results of egg-shell gypsum (E) and plast (M) with the controls (K) and the tendencies resulting from this

week the values of (E) and (K) have greatly decreased (resorption); the values of (M) are considerably higher.

Group II, marrow callus. The high initial values decrease completely by decomposition of the material, running parallel up to the twelfth week.

Group III, connective tissue proliferation in the marrow. High initial values for (M), (E) and (K). Whilst (E) and (K), running parallel to the decline, continually decrease up to the twelfth week, the connective tissue

Time	Stopping	I	II	III	IV	V	VI	VII	VIII	IX
										
2 weeks	E	1·16	*1·03*	0·33	*−2·32*	*1·60*	*1·95*	−0·36	*−1·59*	*−1·24*
	M	0·14	*−0·04*$^{+}$	0·17	*0·12*$^{++}$	*−1·04*$^{++}$	*0·46*$^{+}$	−0·06	*−0·39*$^{++}$	*−0·30*$^{+}$
3 weeks	E	0·91	0·41	*0·59*	−0·04	−0·26	1·33	*−0·35*	*−1·28*	*−1·48*
	M	6·70	0·05	*−0·52*$^{+}$	−1·14	0·04	0·92	*−1·04*$^{++}$	*0·09*$^{++}$	*−0·30*$^{+}$
4 weeks	E	*1·23*	0·26	1·38	−1·05	2·04	1·49	−0·76	*−0·69*	*−1·64*
	M	*0·59*	0·23	−0·29	−0·33	1·78	1·54	−1·54	*0·64*$^{++}$	*0·09*$^{++}$
6 weeks	E	0·39	0·23	1·14	−0·55	*2·88*	*−0·43*	*−0·12*	0·07	0·01
	M	4·29	0·57	1·18	−0·21	*1·14*$^{+}$	*2·23*$^{++}$	*−0·81*$^{+}$	−0·22^{++}	−0·33
12 weeks	E	*0·28*	0·00	*0·36*	−0·26	−0·62	*0·00*	*−0·40*	*0·24*	*0·36*
	M	*1·55*$^{++}$	0·11	*1·85*$^{++}$	−0·36	−0·21	*2·17*$^{++}$	*−1·28*$^{++}$	*−0·65*$^{++}$	*−0·26*$^{+}$

FIG. 6. Groups I to IX. Comparison of the results of egg-shell gypsum (E) with plast (M). Numbers in italics are significant and indicate by + the margin of error up to 10% and by ++ up to 5%

proliferation of (M) in the marrow area proceeds almost constantly up to the twelfth week.

Group IV, external callus. Equal initial values in (M) and (K) which, after a small divergence by falling off in (M), reach their peak between the third and sixth weeks and decrease slightly up to the twelfth week. The reactions of (E) are temporally later and remain parallel below the values of (K) and (M) up to the end of the time of observation.

Group V, apposition callus. The values of (E) and (M) differed distinctly compared with that of (K). (E) is highest in the beginning and (M) is lowest; in both almost the same increase can be observed in the fourth and in the sixth weeks which is followed by an almost parallel decrease up to the twelfth week (resorption). The values of (K) are fairly constant.

Group VI, connective tissue in the drill canal. High initial values for (E), (M) and (K). After a decrease of (M) and (K) up to the fourth week, a rise of (M) follows to the end of the observation time, and a further gradual decrease of (K) up to the twelfth week. The initially great rise of (E) is followed first by a steep decrease up to the twelfth week, parallel to (K).

Group VII, marrow substance in the drill canal. Small initial values for (E), (M) and (K). The greater rise up to the twelfth week is first observed in (K), then in (E), whereas practically nothing can be observed in (M). The great constant connective-tissue reaction by (K) prevents the marrow substance from growing out.

Group VIII, osteoid tissue in the drill canal. Rising values in (M), (K) and (E). In the twelfth week there is almost an equal height in all the values due to resorption processes.

Group IX, density of the osteoid tissue in the drill canal. In the main the graph is to be compared with column VIII. Density increases of all three drillings at the end of the observation time.

Figure 4 shows the arithmetical mean of the original values of groups I, II and IV, i.e. of the callus groups. After a general rise, particularly of (M), the latter is horizontal up to the twelfth week. On the contrary, (E) and (K) are characterized by a slight decrease.

Figure 6 shows a comparison of the plombed drill holes (arithmetical mean from the values of the three drill holes) whith those of the control side.

Group I, inner callus. A decreasing tendency is noted in egg-shell gypsum (E). The initial inner-callus production decreases in the sixth week; an approximately equal production is reached in callus production at the E-plomb and the control side. A rising tendency is noted in metacrylate (M).

Both filling materials show an inversion as to the expansion of the inner callus. The defects plombed with metacrylate showed a significantly greater production of callus compared with the E-plombs at the end of the observation time.

Structural reconstruction of the inner callus could not be observed in any of the cases investigated, but in all the cases, both in plombs and on the control sides, vast resorption phenomena were present at the callus.

Group II, marrow callus. A decreasing tendency is noted in the egg-shell gypsum group. The image of the initial filling with callus and its degradation of the marrow area are congruent with the production of the inner callus. First a rising, then a decreasing tendency is observed in the metacrylate group. The filling of the marrow area is only slightly greater on the plombed side than on the control side. On comparing the defects, we find an initially greater expansion of the callus in the marrow area in the egg-shell gypsum plombs, whereas in later stages no essentially different reaction can be observed between the two plombing materials.

Group III, connective tissue proliferation in the marrow area. A rising, then a decreasing tendency is observed in the egg-shell gypsum group. The proliferation of the connective tissue in the marrow area increases up to the sixth week due to inflammation stimulus, and decreases again with the resorption process. The reactions in the plombed defects are greater than in the control drillings. First a constant, then a rising tendency is observed with metacrylate plombs. With progressing resorption, the equally profuse proliferation which first involves the connective tissue significantly decreases in the twelfth week for metacrylate.

Group IV, external callus. There is a rising negative tendency in egg-shell gypsum. At first the expansion of the external callus is essentially greater on the control side than on the plombed one. An equal production is observed in the twelfth week. No temporal tendencies are noted for metacrylate. On comparing the two plombing materials, we find that the plast has significantly more influence on callus production in the second week. In all the later stages the reaction to the two plombing materials is no longer different. Possibly the egg-shell gypsum mixture is resorbed in this area, in contrast to the splintered polymerisate, by which the production of appositional callus is stimulated outside the drill hole.

Structural reconstruction of the external callus could in no case be observed up to the twelfth week, but distinct resorption phenomena were present.

Group V, apposition callus. First a decreasing, then a rising and, finally, a descending tendency to an equal level were observed for egg-shell gypsum. The reactions on the control side were almost the same in the third week, in contrast to those of the first, fourth and sixth weeks. All were on the same level in the twelfth week due to the resorption processes.

No temporal tendencies were noted between plomb and control for metacrylate fillings. The apposition callus outside the drilling is significantly greater under the influence of egg-shell gypsum in the second and sixth weeks compared with the control. The reactions of the two plombing materials are approximately equal. The reason for the callus growing at a distance from the drill canal can be explained by stimuli of foreign body or inflammation, by plomb material washed away subperifascially, suture, blood coagula transformed fibrinously, etc.

Group VI, connective tissue in the drill canal. There is a decreasing tendency with egg-shell gypsum plombs. The initially existing proliferation of the connective tissue as a reaction to the inflammation stimulus, intensified by the plombing material, subsides again after the fourth week. An equal level is reached in the twelfth week.

There is a rising tendency with metacrylate plomb. The filling of the drill canal with connective tissue is significantly greater compared with the control on the second week with egg-shell gypsum plomb. The reactions are greater with the plast plombs in the sixth and twelfth weeks. Presumably the egg-shell gypsum mixture has already been absorbed by this time in contrast to the plast; this explains the prolonged effect of the metacrylate.

Group VII, marrow substance in the drill canal. No temporal tendency was noted with the egg-shell gypsum compared with the control. There is a decreasing negative tendency with metacrylate. The infiltration of the drill canal with marrow substance is greater in the control part; blocking by connective tissue is present here as on the plombed part. The filling of the drill canal with marrow substance was significantly greater in the second, sixth and twelfth weeks with egg-shell gypsum plombs compared with the control and the plast. Probably since the egg-shell gypsum mixture is resorbed more rapidly than the plast, the marrow tissue may grow out better than in control drillings.

Group VIII, osteoid tissue in the drill canal. An increasingly negative tendency is noted with egg-shell gypsum fillings. The production of osteoid tissue in the control drilling is greater in the plombed part in the beginning, and then it reaches the same level. With metacrylate, there were no tendencies concerning time between plomb and control. The filling of the drill canal with osteoid tissue is significantly greater compared with the control and the plast in the second, third and fourth weeks, and for egg-shell gypsum in the twelfth week. The splintered polymerisate is supposed to be released and then washed away with the increasing resorption of the osteoid tissue. Thus, our impression is that the effect of the egg-shell gypsum mixture is distinctly greater in the twelfth week than that of the plast. However, this is relative due to the great difference compared with the control value.

Group IX, density of the osteoid tissue in the drill canal. An increasingly negative tendency is noted with egg-shell gypsum plombs, but no tempor a

tendency in the plomb and the control with metacrylate. The assessment of the two sides is almost the same. The density of the osteoid tissue shows the same picture as the filling of the drill canal with osteoid tissue. Thus, the density of the osteoid tissue in the drill canal is to be judged compared with the control. The same significances have been noted here.

If we add the significant values of egg-shell gypsum and metacrylate plombs, we obtain a ratio of E 11 : M 11 or of E : M = 1 : 1. Since the degree of the interdependence of the evaluations is not certain and is connected with no qualitative evidence, this calculation can only be informatory. During our observation period, the single evaluation groups were more or less influenced by two filling materials in the regeneration process. A comparison of our results obtained with rabbits with those of Tarsoly and Tomory (1963) and Bornemisza and Bakó (1958) with dogs seem to be premature at present. In contrast to the authors mentioned, we made smaller defects in all our drillings which seem to have a different manner of regeneration in the relatively thinner compacta and in the statically far less loaded rabbit tibia than in the tibia and femur of the dog.

Our groups I to IX give a quantitative and topographical insight into the tissue reactions in bone regeneration under the given experimental conditions. It is a fact that other forms of evaluations may extend and complete the picture. Bone regeneration is a complex process which cannot be considered and judged from one-sided points of view. An experimentally influenced bone regeneration cannot be judged by single findings, but has to be verified by as many different methods as possible and in a larger animal material comprising different species.

SUMMARY

On rabbit tibiae drill defects of different sizes were filled up with egg-shell gypsum or splintered polymerisate (Piacryl DF z) of a grain size of $< 60\ \mu$ and the bone regeneration was histologically investigated. The assessment of the bone formation or regeneration process, performed according to topographical, quantitative criteria, shows significant influences by the filling materials during an observation period lasting 2 to 12 weeks. No general influence on bone regeneration could be observed in the mechanically slightly loaded rabbit tibia either when egg-shell gypsum or splintered polymerisate was used for filling up the defects.

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Symp. Biol. Hung., 7, pp. 251—256 (1967)

CLINICAL RESULTS REGARDING THE FILLING UP OF BONE CAVITIES WITH A MIXTURE OF EGG-SHELL POWDER AND PLASTER

by

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Cavities may develop in bones as a result of inflammatory, tumorous and pathological metabolic processes. Several authors have given accounts of the significance and manner of filling in such cavities after excochleation; these, however, are not at all new concepts. The debate centres on the material with which to fill in the cavity.

As autoplastic or homoplastic material, the spongiosa or cortical piece obtained from the hip blade or the rib proved very suitable (Kyselka 1960, Borsay and Varró 1959, Nicoll 1956, Hadrava 1959, Hellner 1958, Schnitzler 1960, etc.). The assessment of the results obtained with the "Kiel bone" is still carried out both in Hungary and abroad; numerous scientists employ it clinically or are occupied with the technology of its production (Bauermeister 1958, Kondrai 1964). The tendency to reduce surgical intervention, the difficulties of producing and obtaining the preserved bone (the lack of a bone bank) focussed attention on heteroplastic materials. A number of authors (Fründ 1954, Orell 1951) already gained experience in this field, and their studies inform us of the results obtained by using the most diverse materials (plaster, oils, synthetic material).

On the basis of Krompecher's experiments (1958, 1959), — according to which pulverized egg-shell has an osteogenetic effect — we tried to fill in bone cavities with pulverized egg-shell in our experiments with animals, thus, departing from the use of customary materials. For the sake of attaining better plasticity (pulverized egg-shell in itself mixed with water sets with difficulty to become mouldable), the pulverized egg-shell was mixed 1 : 1 with plaster. Experiments were carried out on 10 dogs. Cavities with diameters from 10 to 15 mm were chiselled in thick, tubular bones and then filled in closely with the above mixture. Developments were observed histologically and the favourable ossifying effects of our mixture were demonstrated (Tarsoly 1963, Tarsoly and Tomory 1963). Undoubtedly, plaster cannot be considered merely as a carrier-material, for the favourable results of its use have been dealt with as already mentioned. For the purpose of control, plaster was employed alone, and then mixed with pulverized egg-shell in an experiment under similar conditions. The ossifying effect

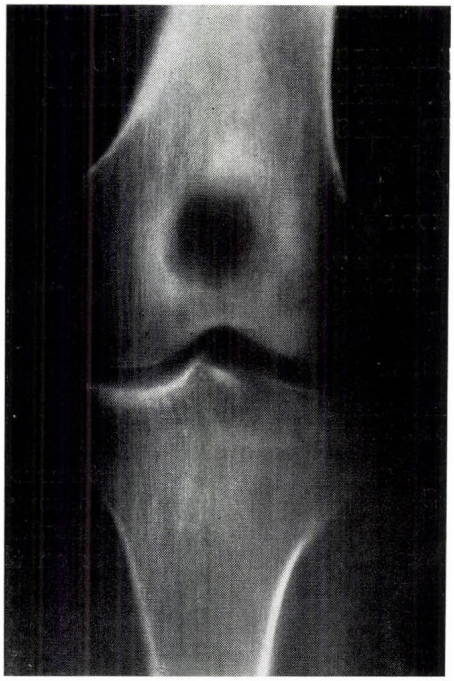

FIG. 1. Hazel-nut sized rarefaction surrounded by sharply contoured calcified zone in the distal epiphysis of the femur

of the egg-shell-powder-plaster mixture considerably exceeded the result obtained by using merely plaster. Consequently, on the basis of the results obtained in our experiments, we felt justified in trying out the procedure in clinical practice. This was done as follows.

The sterile plaster and the egg-shell powder were prepared in advance for the operation in a small Petri dish. The plaster was sterilized in dry hot air, and the egg-shell powder was disinfected with Sterogenol according to Krompecher. The cavity was exposed through as small an opening as possible at a spot where the cortex was thinnest. The contents of the cavity was cleaned out and the cavity was filled in with gauze soaked in hot saline to check bleeding. This we consider extremely important, because subsequent bleeding softens the mixture when the cavity is being filled in and is, therefore, disadvantageous.

While checking the bleeding there is time to mix the two ingredients by adding saline + antibiotic in such a ratio as to enable us to obtain a well mouldable mass similar to that of plaster pulp. A thin, pencil-like bar can easily be formed with our fingers from the pulp which is then placed into the desanguinated cavity with suitable instruments the way a dentist performs the stopping of a cavity. Before filling in the cavity, however, the soft parts should be kept clear of the opening

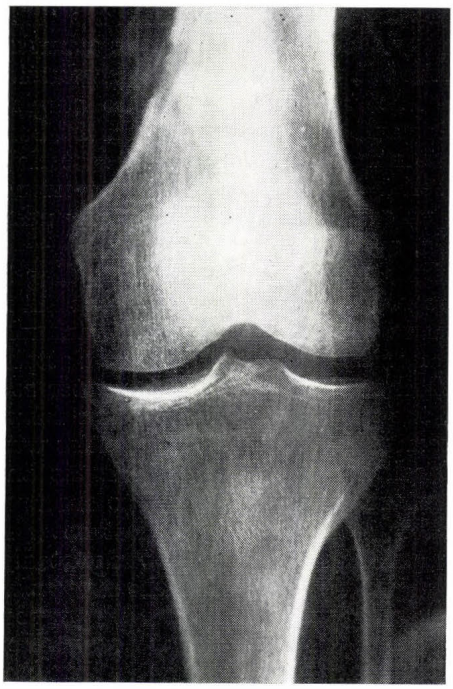

FIG. 2. Cavity filled up with the mixture

FIG. 3. Result of control examination performed after one year

of the cavity or even isolated. Experience has shown that the dilute pulp diffusing into the soft parts retards the healing of the wound. The cavity can be closed perfectly if the opening of the filled cavity can be stopped cork-wise with the cortical piece obtained while exposing the cavity. Before applying the egg-shell-powder-plaster mixture, the cavity may be flushed with an antibiotic solution. Spraying with powder is not recommended because it stimulates bleeding. We describe two cases below as illustrations.

1. D. M. a 53-year-old woman was admitted for treatment who had been suffering from knee complaints for years. In her X-ray picture a hazel-nut sized rarefaction was observed in the epiphysis of the femur above the knee surrounded by a sharply outlined calcified zone (Fig. 1). The alteration which proved to be chronical osteomyelitis histologically, was cleansed and the cavity was closely filled in with our mixture (Fig. 2). The wound healed *per primam* and the patient was dismissed two weeks after the operation. A year later the control examination showed that the movements of the joints were free and the patient had no complaints. The X-ray picture showed a complete reconstruction of the cavity (Fig. 3).

2. B. T. a 36-year-old man complained of pain in his left foot, and his complaints did not cease after symptomatic treatment. On admis-

FIG. 4. Hazel-nut sized sharply contoured cavity shadow in the anterior part of the left calcaneus

FIG. 5. Control X-ray picture after 3 months

sion the X-ray picture showed a hazel-nut sized, sharply defined cavity shadow in the projection of the anterior part of the left lateral calcaneum (Fig. 4). On exposing the alteration, serum was extracted from the cavity, and after cleaning the wall of the cavity of the grayish-red granulation, a mixture of egg-shell powder was applied. Histologically the alteration proved to be a bone cyst. The filling in of the cavity, the absorption of the mixture can be followed very well in the X-ray pictures (Figs. 5 and 6).

A total of 7 patients were treated so far with egg-shell powder implantation. This relatively small number does not permit far-fetched conclusions to be drawn of the value of our procedure. However, it may be ascertained that the experience gained so far with this exceedingly cheap and technically easily producible, immunbiologically inactive mixture has so far lived up to our expectations on the ground of the conclusions drawn from animal experiments. On assessment we had to take into consideration the instructive healing results obtained by filling up the bone cavity with other material. Our institute is in possession of 10 years' experience in the field of healing cavities with auto- and hetero-transplants of spongious and cortical bones. *It has generally been observed that the cavities fill up, but osteoclasia does not ensue even years later, though it is true that the*

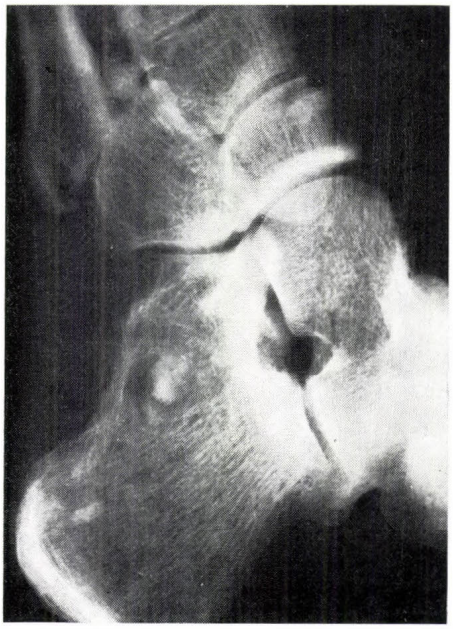

FIG. 6. Control X-ray picture after 6 months

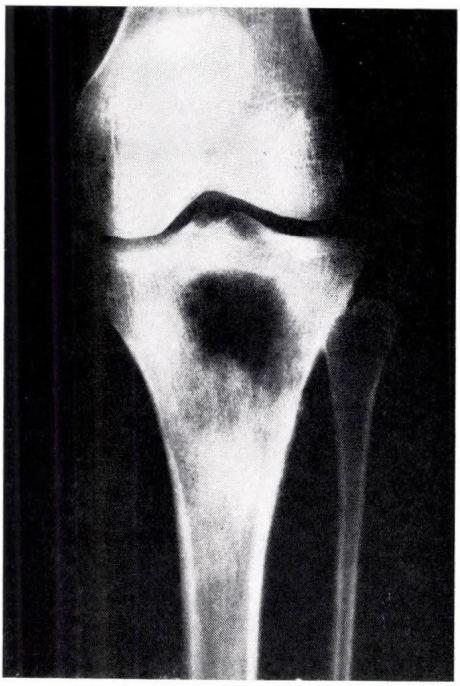

FIG. 7. Nut-sized cavity in the proximal epiphysis of the tibia

patient does not suffer any serious consequences clinically on account of this.

3. E. T. In the region of the metaphysis of the left tibia of a 30-year-old woman a nut-sized cavity of an aspecific osteomyelitis origin was filled in with rib fragments (Fig. 7). Two years later the implanted cortical pieces could still be seen in the X-ray picture (Fig. 8). The patient has no complaints and the joint is capable of unobstructed movement.

In contrast to the bone fragments, after the employment of our mixture we were able to observe almost perfect anatomic healing in several cases months later. We intend to continue the clinical application of our procedure and to perfect its technique on the basis of the results obtained hitherto.

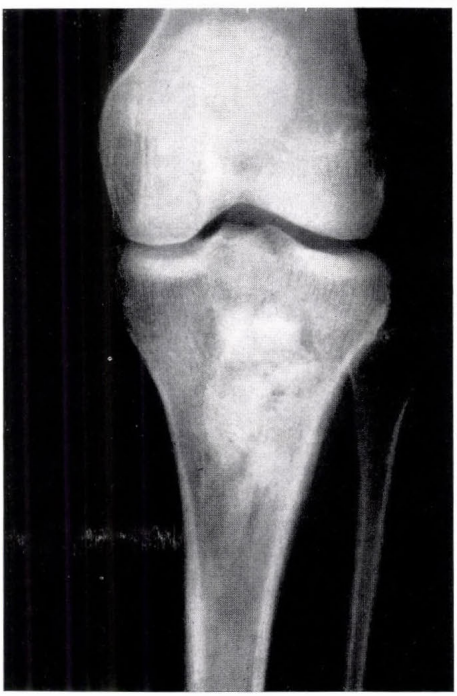

FIG. 8. Control X-ray picture after 2 years

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Symp. Biol. Hung., 7, pp. 257–262 (1967)

EXPERIMENTS ON ISOLATED CHONDROCYTES

by

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In mature cartilage about two-thirds of the tissue volume is occupied by the cartilage matrix (Bloom and Fawcett 1962). This fact seems to suggest that the matrix significantly influences the behaviour of the tissue. However, a large amount of matrix is also a serious hindrance in experiments on the cartilage cells. At least some of these difficulties in experiments on chondrocytes might be avoided by liberating the cells from the matrix. Bearing this in mind, we attempted to isolate chondrocytes. Two essentially different methods of chondrocyte isolation were developed. One in which a small number of cells can be isolated from already fixed cartilage; this material was used for experiments on the localization of sulphated mucopolysaccharide synthesis. For this purpose some special microchemical estimations were performed. The other isolation method, based on enzymic treatment of cartilage sections, gives millions of living and functioning cells. These cells were used for transplantation experiments.

The site of sulphated mucopolysaccharide synthesis in cartilage cells. The cartilage matrix is presumably in a state of constant renewal. This was studied in experiments on intracellular production of sulphated mucopolysaccharides of cartilage. The presence of sulphated mucopolysaccharides was demonstrated in isolated chondrocytes by the micromethod developed for the $\mu\mu$l range (Kawiak 1963a, Kawiak and Kowalski 1964). For isolation of chondrocytes, the deparaffinized paraffin section from fixed material was placed in 80% ethanol or in water, and then the section was removed with a needle (Fig. 1). The chondrocytes of the opened lacunae, adhering to the glass surface remained on the slide, while the cartilage matrix was removed. The isolated cells were grouped with the micromanipulator. All further biochemical manipulations were performed likewise under the microscope with the aid of a micromanipulator. Separated groups of 20 cells were extracted by alkali (0·5N NaOH, 4° for 20 h) or papain (at room temperature 2 × 24 h). The extracts obtained were neutralized, and then the mucopolysaccharides were precipitated with rivanol according to Whitehouse and Boström (1961) and Moretti and Whitehouse (1963). Only sulphated mucopolysaccharides were found in chondrocytes, and about 10 to 40 $\mu\mu$g of chondroitin sulphate equivalent per cell was estimated.

In view of these results, it was interesting to test if sulphated mucopolysaccharides could be synthesized or stored in some restricted region of the

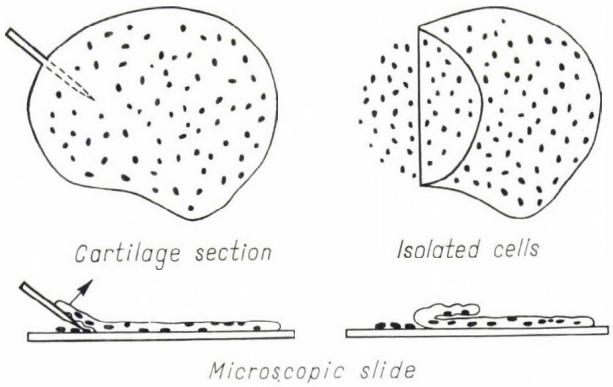

FIG. 1. Scheme of the isolation procedure of chondrocytes from a section of cartilage

cell. Presumably, the sulphation process of sulphated mucopolysaccharides can be taken as an indication of synthesis of the whole polysaccharide molecule *in vivo*, although *in vitro* the possibility of sulphation without the synthesis of the polysaccharide chain was demonstrated (Adams 1952). This phenomenon was utilized in experiments on rat epiphyseal cartilage which was incubated in the presence of sodium sulphate [^{35}S] (Amersham) at 37° only for a short period (10 min). Within this time the isotope was already incorporated into the limited region of chondrocyte cytoplasm (Kawiak 1963b; Fig. 2). From autoradiographic observations on chondrocytes released from the cartilage matrix, their synthesis or the storage of already polymerized mucopolysaccharide(s) in the Golgi region of the cartilage cell was suggested (Kawiak 1963b). Godman and Lane (1964) confirmed this suggestion by electron microscopy.

Isolation of living chondrocytes. Besides the dead cells, also the living and functioning chondrocytes can be liberated from cartilage. Living chondrocytes were isolated from a matrix of nasal septa cartilage of calves and rabbits (Kawiak et al. 1965). To secure uniform liberation of cells from the matrix, the cartilage was cut into 120 μ sections with a microtome. Isolation of cells was conducted in a weighing bottle divided into two compartments by a nylon filter and containing a metal rod sealed in a glass tube. The cartilage sections were initially treated with Hanks solution containing trypsin at 37° for 20 min. During this time the content of the weighing bottle was stirred on a magnetic stirrer. Then the enzyme solution was discarded and replaced by Hanks solution containing trypsin and collagenase. The second portion of the enzyme solution was stirred for 20 to 45 min, and at the end of this period practically all the cartilage sections were dissolved. The suspension of liberated cells withdrawn from the lower compartment of the vessel was filtered through nylon mesh. The cell suspension was overlayered on 0·25M saccharose solution and the cells were sedimented at 100 g for 5 min. One to three millions of cells were obtained in a single procedure from 1 g of fresh tissue. The cells were viable according to tests performed with trypan blue and neutral red. However, some impairment

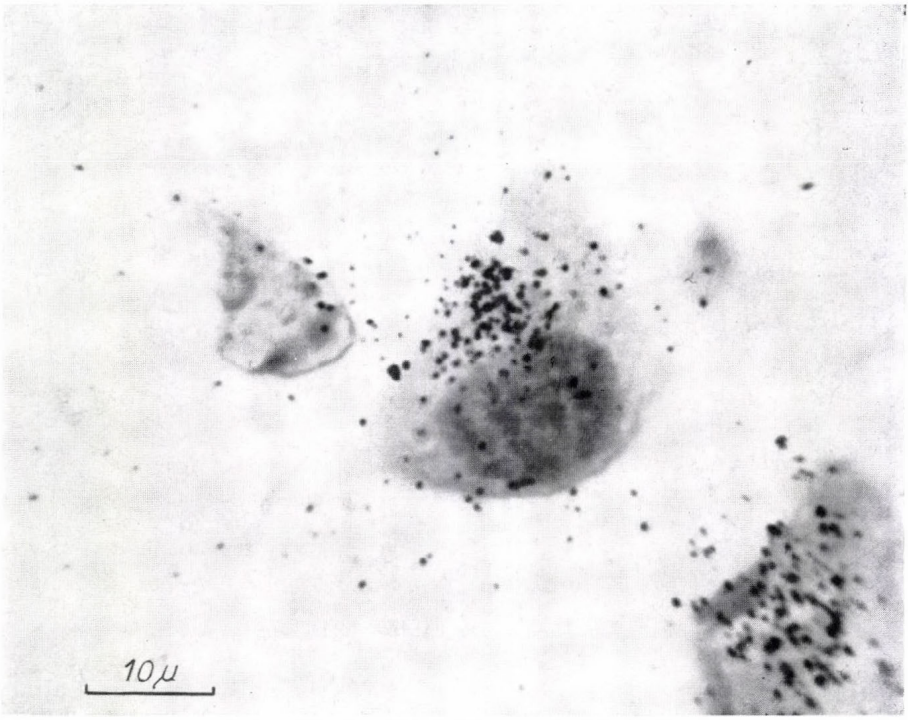

FIG. 2. Autoradiograph demonstrating the localization of sulphation in chondrocyte derived from rat femoral epiphysis

of ^{35}S incorporation into the cells was noted, and prolonged treatment with Hanks solution led to the appearance of 'bubbles' on the cell surface.

Production of cartilage matrix by chondrocytes isolated from mature cartilage. The ability of synthesis of the cartilage matrix, as indicated by the incorporation of ^{35}S into chondroitin sulphate (Dziewiatkowski 1951) or after partial removal of the matrix by papain injection to rabbits (Engfeldt and Westborn 1960, Thomas 1956), persists in adult animals. However, it is not clear if cells obtained from mature cartilage at the stage when no matrix surrounds them, will be able to restore the matrix. In order to answer this question 3·7 to 14 $\times$ 10^5 chondrocytes isolated from female rabbits were injected with a syringe into one of the tibial anterior muscles of a male rabbit. The cells obtained from the same isolation, frozen and thawed five times in order to kill them before transplantation, served as controls. They were injected into the muscle of the animal's other leg. The recipient rabbits were killed 7, 14, 28 and 42 days after transplantation and the whole tibial anterior muscles were removed, fixed and examined histologically and histochemically.

Rabbit septal cartilage had the structure of hyaline cartilage (Fig. 3). Chondrocytes contained in their cytoplasm one or more vacuoles, frequently

of considerable size. Lipid droplets corresponding to the vacuoles were seen in Sudan IV stained sections. Sex chromatin was present in 74 to 78% of female and 8 to 12% of male chondrocytes (see table below).

Incidence of sex chromatin in nuclei of chondrocytes from septal cartilage and from cartilage formed after transplantation of isolated female chondrocytes

Type of cartilage	No. of specimen*	Nuclei containing sex chromatin (%)
Male septal cartilage	1	8
	2	10
	3	12
Female septal cartilage	1	78
	2	74
	3	76
Transplants	1	78
	2	77
	3	71

*Note** = in each specimen 100 nuclei were counted.

Isolated cells were spherical or oval in shape. A large number of cells contained lipid droplets in the cytoplasm (Fig. 4). The transplanted chondrocytes were localized in a muscle or in the perimysium. The cartilage usually assumed an elongated shape in the direction of the canal formed by the needle during injection. In seven-day transplants immature cartilage was present. The cartilage matrix was scanty and fibrillar in appearance and gave a metachromatic reaction, particularly in the vicinity of cells. Chondrocytes were arranged close to one another and contained lipid vacuoles. More intercellular substance was observed after 14 days, and after 28 and 42 days the cartilage of transplants assumed an appearance resembling the septal cartilage used for isolation (Fig. 5). The chondrocytes contained large lipid vacuoles and 71 to 78% of them contained sex chromatin in the nuclei (see table). In control experiments, in which dead cells were injected, no sign of cartilage formation could be found.

Induction of bone and cartilage is a frequent phenomenon in the rabbit (Bridges and Pritchard 1958). Bone induction following injection of tissue extracts into rabbit muscles was also reported (Hartley and Tanz 1951, Heinen et al. 1949). Therefore, cartilage induction should be distinguished from the formation of new matrix by the transplanted cells. We did not, however, observe cartilage formation in transplants of dead cells injected in the same number and from the same isolation as the transplanted living cells. Moreover, the natural labelling of cells by sex chromatin (Moore and Barr 1954) suggests that the injected cells surrounded themselves by cartilage matrix. Besides, many chondrocytes in the septal cartilage and in

FIG. 3. Rabbit nasal cartilage. Hematoxylin-eosin; × 200

transplants contained a large lipid droplet in the cytoplasm. All these facts taken together indicate that the cartilage matrix was formed by transplanted chondrocytes, and not as a result of induction.

Transplants examined one month or more after implantation were surrounded by cellular infiltration. This observation suggested some sort of immune response to transplanted cells. Thus, some further experiments were planned on the immunological response to cartilage transplants.

Role of cartilage matrix in immune response of cartilage transplants. It is a known fact that cartilage transplanted from one animal to another within the same species is not rejected as, e.g., skin grafts (Craigmyle 1958, Schatten et al. 1958). This unusual behaviour of the cartilage can be connected with the presence of cartilage matrix which separates the chondrocytes from the immune competing cells and in this way prevents the transplant from destruction. Presumably, the cartilage matrix is not an antigen against the cartilage cells, and the cells can constantly renew the components of the matrix. The validity of this hypothesis could be verified in experiments with transplantation of isolated chondrocytes.

Keeping this hypothesis in mind, a nasal cartilage was surgically removed from a rabbit under anesthesia. For simplicity, this rabbit will be denoted *A*. From another rabbit, *B*, nasal cartilage was also removed. Small fragments of cartilages *A* and *B* as well as chondrocytes obtained from cartilage *A* and the cells from cartilage *B* were separately transplanted on the back of rabbit *A*. Preliminary results suggest that the whole autologous *A* cartilage as well as cartilage formed by injected chondrocytes *A* are not surrounded

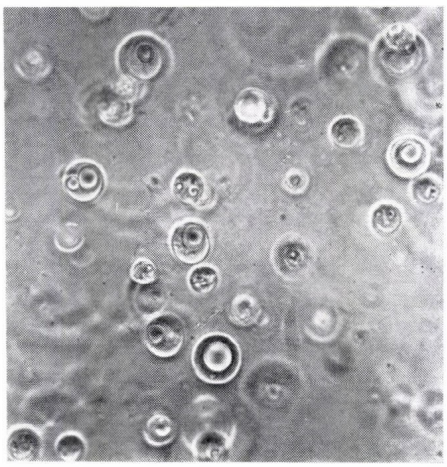

FIG. 4. Chondrocytes isolated from rabbit nasal cartilage. Phase contrast, living; × 400

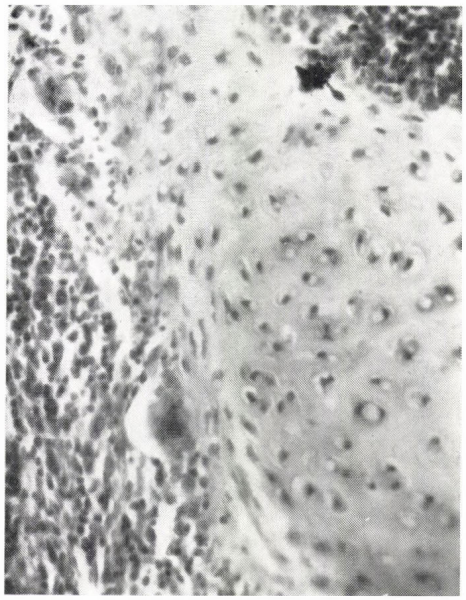

FIG. 5. Cartilage formed 28 days after transplantation of isolated rabbit nasal chondrocytes. Haematoxylin-eosin; ×400

by infiltrating cells, in contrast to the fragment of cartilage *B* and the newly formed cartilage from cells *B*. Although the preliminary results roughly agree with the hypothesis presented above, it is too early to draw any conclusions from these experiments.

SUMMARY

Two methods for isolation of chondrocytes are described. One in which already fixed cells can be liberated from the cartilage matrix; the cells isolated in this way were used in experiments on the intracellular localization of the synthesis of sulphated mucopolysaccharides. The other isolation method, based on enzymic treatment of living cartilage, liberated living and functioning chondrocytes. The cells isolated from mature cartilage form a new cartilage matrix in the recipient animal.

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Symp. Biol. Hung., 7,pp. 263—268 (1967)

ON THE BIOSYNTHESIS OF MUCOPOLYSACCHARIDES

by

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THE METABOLISM of mucopolysaccharides (MPS), their analysis and their synthesis is of considerable interest biochemically and clinically, because they form important constituents of body fluids and body tissues. The tasks they have to fulfil, e.g. in the connective and supporting tissues (chondroitin sulphates A, B, C), with blood coagulation (heparin) or as substances specific for blood groups (fucomucines) are to be considered. These compounds are of growing importance with regard to immunochemical processes with bacterial and virus infections. Callus formation is a reparative process accompanying the development of MPS. Lindner (1964) recently pointed out that the so-called synthetic phase with healing wound begins with the formation of the ground substance which contains the MPS and which probably controls and regulates the fibre development as a kind of matrix. Thus, the biosynthesis of the MPS is of considerable interest in callus formation.

MPS are substances of heterogenous structure which exist in a free or bound form as do mucoproteins or glycoproteins. Meyer distinguishes between acid and neutral MPS, and subdivides the acid MPS according to their structure into simple and complex acid MPS. All the MPS, including the neutral ones, contain amino-sugar as a common constituent. Besides these amino-sugars, uronic acid and acetic acid can be found in the simple acid MPS; the complex MPS give sulphuric acid under hydrolysis. Quite recently compounds have been repeatedly isolated that contained a neuraminic acid as an acid component. The neutral MPS consist of an amino-sugar and varied quantities of other monosaccharides, namely glucose, galactose, mannose and fucose.

A consideration of the biosynthesis of the MPS includes all these elements of the MPS. Among them, the elements of the acid MPS will be more closely discussed. Our knowledge of the biosynthesis of the MPS has been chiefly obtained during the last ten years. This progress has been facilitated by improved methods of preparation and identification of the MPS and their metabolites and by the isotope technique.

Glucuronic acid and iduronic acid, an isomeric compound of the glucuronic acid, are, besides glucosamine and galactosamine to be considered as basic elements of the acid MPS. In certain cases derivatives of neuraminic acid are to be added. This, however, will not be discussed here.

Experiments on *Streptococci* with labelled monosaccharides have shown that glucose in particular must be considered as a precursor of amino-sugars and of glucuronic acid. Galactose and fructose prove to be less efficient. The way to the most important elements of the MPS appears to be via glucose. To reach the hexuronic acids mentioned, an oxidation has to take place at the C-atom 6 of the glucose molecule. It is known today that this oxidation does not take place at a free glucose molecule but at a nucleosid-glucose-compound. The latter has been described by a team of Leloir in 1950 (Caputto et al. 1954). The nucleosid-glucose-compound, as demonstrated by Munch-Petersen et al. (1953), develops by the reaction of the mononucleotid uridine-triphosphate (UTP) with a glucose compound rich in energy, the glucose-1-phosphate. During the separation of pyrophosphate uridine-diphosphate (UDP) glucose develops:

$$\text{UTP} + \text{glucose-1-phosphate} \rightarrow \text{UDP-glucose} + \text{PP}.$$

As is known, glucose-1-phosphate is a link in the intermediary carbohydrate metabolism and is sufficiently available. With UDP-glucose, glucose is present as a compound active in metabolism, as active glucose. Generally, it plays an important part in the intermediary metabolism of carbohydrates. The active glucose molecule may enter into a multitude of reactions. We shall mention those that have a relation to the MPS-synthesis. Here the oxidation of glucose towards glucuronic acid should be mentioned. According to the investigations of Strominger et al. (1954), this reaction may be catalysed by an enzyme of the liver and needs the niacinadenin-dinucleotid (NAD) as hydrogen acceptor. The reaction may be formulated as follows:

$$\text{UDP-glucose} \xrightarrow[\text{NAD} \rightarrow \text{NADH}_2]{\text{liver enzyme}} \text{UDP glucuronic acid}.$$

During this reaction the glycosidic hydroxyl group of the C-atom 1 of the glucose is protected from oxidation by its connection with the rest of the uridyl. The developed UDP glucuronic acid now serves as a group carrier and may be built into the MPS mentioned. Thus, the way from glucose to glucuronic acid is laid open. Iduronic acid, a component of the chondroitin sulphuric acid B (skin) develops by epimerization at the C-atom 5 of the glucuronic acid, owing to the participation of an epimerasis. This transformation has recently been pointed out by Jacobson and Davidson (1963). Whether a transformation of UDP-glucose into UDP-idose takes place is not yet verified but may be considered possible.

Another reaction of the UDP-glucose which is of interest is its transformation into galactose. Principally, it is an epimerization, this time at the C-atom 4 as is shown with the development of iduronic acid. Thus,

$$\text{UDP-glucose} \xrightarrow{\text{C 4 — epimerasis}} \text{UDP-galactose}.$$

Besides this reaction, another, under co-operation of a transferase, has been described:

Galactose-1-phosphate + UDP-glucose $\xrightarrow[\text{(glucose-1-phosphate-uridyltransferase}]{\text{transferase}}$ UDP-galactose + glucose-1-phosphate.

It may be supposed that by these reactions sufficient galactose will be available for the development of the amino-sugars needed for the composition of the MPS. According to Gibian (1959), the latter may develop by the direct addition of an amino group to galactose or glucose phosphates or by the reaction of fructose-6-phosphate that appears with glycolysis.

Fructose-6-phosphate + NH_3 → glucosamine-6-phosphate + H_2O.

Glutamine is to be mentioned especially as an amino-group donor. Heyns and Koch (1952) were able to demonstrate that fructose, NH_4Cl and phosphate form glucosamine without the participation of enzymes. After transformation of glucosamine-6-phosphate into glucosamine-1-phosphate, UDP-glucosamine may develop which may be transformed into UDP-galactosamine similar to the reaction described. Maley and Maley (1959) confirmed this mechanism of transformation. According to Boström (1960), free glucosamine can apparently be transferred with the co-operation of adenosine triphosphate into glucosamine-1-phosphate and can thus, react with UTP.

The hexosamines appearing in the MPS are in most cases acetylated to the nitrogen atom. With the co-operation of acetyl-coenzym A, both the glucosamine and the galactosamine are transformed into their acetylated derivatives. Both acetylated hexosamines may be mutually transformed into their UDP-compounds (Glaser 1959). Corresponding derivatives of galactose may be formed at various stages of reaction: glucose — galactose, glucosamine — galactosamine, acetyl glucosamine — acetyl galactosamine. The phases of reaction demonstrated hitherto during the development of MPS are fairly well known. Among the following reactions only some are verified by experiments. The question is how the proper chain of polysaccharides is formed and when or at which phase of reaction sulphuric acid is introduced into the sulphate-containing MPS. Boström (1960) considers both questions as the most delicate problems of the MPS-synthesis. Gibian (1959) maintains that disaccharide units are formed in the introductory phase of the synthesis, because, in his opinion, MPS appear only as even-numbered polymers. He formulates the supposed course of reaction as follows:

UDP-glucuronic acid (GS) + → UDP-glucosamine (GA) → GS-(UDP-GA)

→ UDP-acetyl-glucosamine (AGA) → GS-(UDP-AGA)

→ UDP-acetyl-galactosamine (AGaA) → GS-(UDP-AGaA).

It is probable that such a mechanism of reaction exists with the formation of chitin:

$$\text{UDP}-\text{AGA} + \text{UDP}-\text{AGA} \rightarrow \text{AGA (UDP}-\text{AGA}).$$

According to Gibian, a development of disaccharides is also possible via amino-sugar phophates:

UDP-glucuronic acid + acetyl-glucosamine-phosphate → glucuronylacetyl--glucosamine-phosphate.

The disaccharide-phosphate is said to react with UTP, thus, again developing glucuronyl-(UDP-acetyl-glucosamine).

According to the two mechanisms, an active disaccharide would be developed which could equally be an acceptor or donor in polymerization. It is probable that several polymerases take part here. The question of the introduction of sulphuric acid still remains open. There is no evidence of the coupling of disaccharide units to polymer MPS. The biosynthesis of the hyaluronic acid has been investigated (Dorfman 1962) very thoroughly but not completely. *Streptococcus haemolyticus* enzymes develop hyaluronic acid from UDP sugar derivatives. Here two reactions are catalysed:

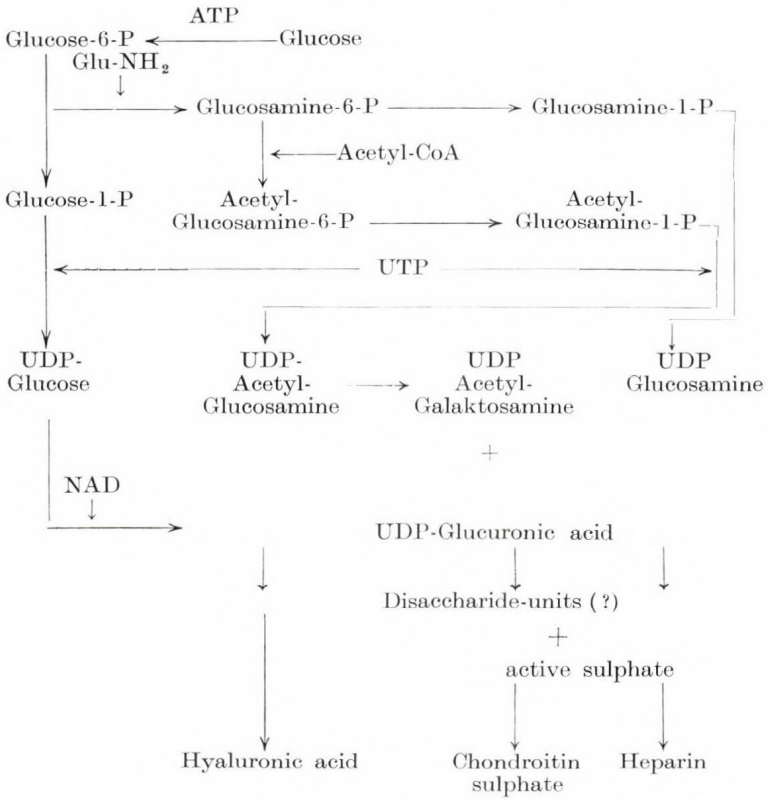
1. The connection of the UDP glucuronic acid with the C-atom 3 of the acetyl-glucosamine;
2. The transfer of acetyl-glucosamine from UDP-AGA to the C-atom 4 of the glucuronic acid.

According to these results, an alternate addition of the single monosaccharides appears to be possible. The question arises at what stage the installation of the sulphates takes place. According to Gibian, sulphating is said to be closely linked to the amination and to take place at an early stage of the MPS-synthesis; probably at the monosaccharide, but at the latest at the disaccharide stage. As proof Gibian mentions that C-14-glucose, C-14 labelled acetate of the carboxyl group and labelled sulphate *in vivo* produce practically identical half-lives, as has been tested with the chondroitin sulphuric acid of the skin and that Strominger (1955) was able to isolate UDP-acetyl-galactosamine-sulphate from the oviduct of laying hens. On the other hand, Delbrück and Lipmann (1959) obtained an enzyme from embryonic calf cartilage that transfers, e.g. sulphate to the chondroitin fraction of the cornea.

It may be considered that both reactions are possible. It is undisputed, however, that the rest of the sulphate is produced by adenosine-3'-phospho-5'-phosphosulphate, the active sulphate. Active sulphate is developed in two phases of the reaction with the co-operation of adenosine triphosphate (ATP)-sulphurylase and ATP-kinase, as demonstrated by Robbins and Lipmann (1956).

In conclusion, a schematic diagram below shows the phases of synthesis that have led to the formation of mucopolysaccharides. This scheme has

been plotted according to the data of Dorfman (1955), Zambotti (1957), Gibian (1959) and Brimacombe and Webber (1964). For the sake of clarity, not all the reactions discussed are indicated.



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Symp. Biol. Hung., 7, pp. 269—273 (1967)

ELECTRIC FACTOR IN BONE REGENERATION. REPORT ON STUDIES *IN VITRO* AND *IN VIVO*

by

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When performing the rehabilitation of patients with recently healed bone fractures, Cieszyński (1963a) noticed that baths in iodic-bromic salines exert either a favourable or a noxious influence, depending on the origin of the saline in use.

It was found that the iodic-bromic saline from Iwonicz-Spa has an advantageous influence (in conformity with Gabryszewski's previous observations), but the iodic-bromic saline of Jastrzębie-Spa appeared to act quite unfavourably in these cases. This striking effect could not be explained either from the chemical or the balneological point of view.

Cieszyński's (1963a) *in-vitro* experiments solved this problem. He showed that both salines differ from each other by the opposite electric surface-depth potential whose extreme values correspond precisely to the therapeutic concentrations of the solutions (Fig. 1).

These measurements made it possible to explain the apparently paradoxical therapeutic effect in conformity with the hypothesis in Kubicz and Cieszyński (1960), assuming that the potential difference in the salinic baths causes the transposition of the loosely bound electrons in the patient's body.

In order to clarify the role of electricity in the regeneration of bone tissue, Cieszyński (1963b) performed further studies on animals. Rabbits were used, in which fractures were produced of both forelimbs. Then the fractured limbs were placed into metal-sheet splints for immobilization. In the artificial cell system one of the limbs was treated with electricity, while the other one was left to heal spontaneously. In the biological cell system both limbs were treated with electricity.

It was found when using the artificial cell system (Fig. 2) that near the positive electrode, on the dorsum of the rabbit, a mineral tumour arose inside the subcutaneous tissue (Fig. 3). At the same time, in the limb which was under the influence of a negative electrode, the regeneration of callus appeared to be slightly depressed in comparison with the control limb in which a more valuable callus was spontaneously formed (Fig. 4).

Some other experiments which were performed according to the layout shown in Fig. 5 revealed that, when influencing the fractured limb with a positive electrode, a callus is obtained which is 1·5 to 3 times stronger than that of the control limb. These mechanical measurements were taken by the method shown in Fig. 6.

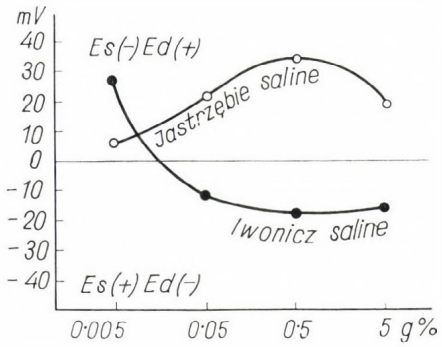

FIG. 1. Electric surface-depth potential of Iwonicz and Jastrzębie salines as a function of concentration of the solution

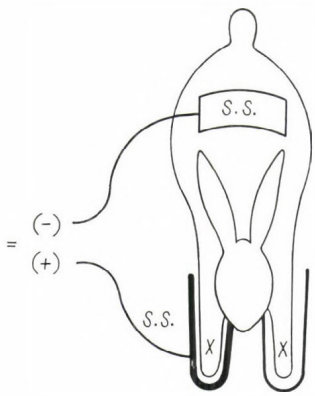

FIG. 2. Scheme of connection of the electrodes in the first experimental group. S.S. = stainless steel; X = site of fracture

Moreover, when examined histologically, it was found that the structure of callus developed near the positive electrode was almost mature and largely regular, while that of the control limb was rather young and irregular.

In a group of experiments the biological cell system was employed as shown in Fig. 7. The radiogram (Fig. 8) shows an abundant almost uniform callus in the right limb which was fixed in an aluminium electrode, whereas in the left limb which was fixed in a stainless steel electrode there is a typical pattern of delayed callus.

The same observations can be made on pictures of the histological specimens (Figs 9 and 10) as on the radiogram.

It has to be emphasized that near the aluminium electrode the positive electric charges, and near the stainless steel electrode the negative ones were concentrated.

FIG. 3. A mineral shadow is visible above the vertebral column of a rabbit whose dorsal region was influenced by the positive electrode according to the scheme in Fig. 2

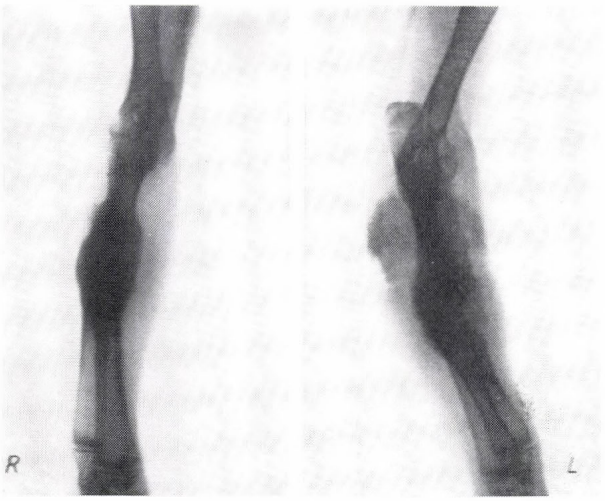

FIG. 4. Radiogram of the rabbit in Fig. 3. In the right fore-limb, a solid bone concretion with smooth edges. In the left fore-limb, a mineral concretion less uniform surrounded by irregular callus tissue

The experiments indicate that positive electricity stimulates callus formation, while negative electricity depresses it. Theoretically, the nature of positive and negative electricity can be explained

(a) by the transposition of the loosely bound electrons and by electrochemical oxidation and reduction reactions;
(b) by the action of "electrons and holes";
(c) by the action of elemental electric charges of both signs (possessing great mobility and some energy)
(d) by the action of non-ionic "electric carriers".

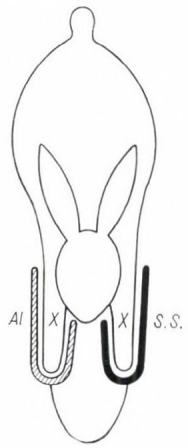

FIG. 5. Second scheme of connection of the electrodes.
S.S. = stainless steel; X = site of fracture

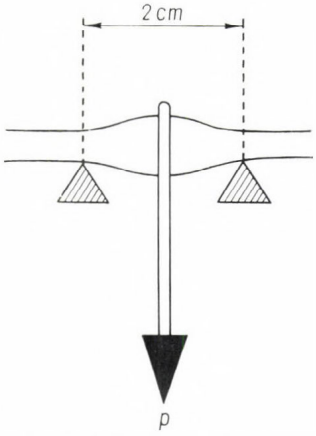

FIG. 6. Scheme of the apparatus for measuring endurance to fracture. P = load force

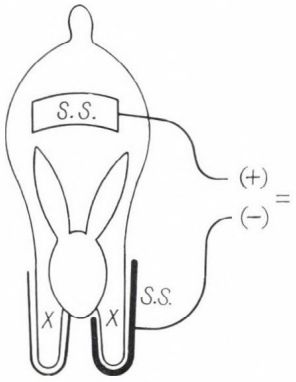

FIG. 7. Scheme of biological cell system. Al = aluminium; S.S. = stainless steel; X = site of fracture

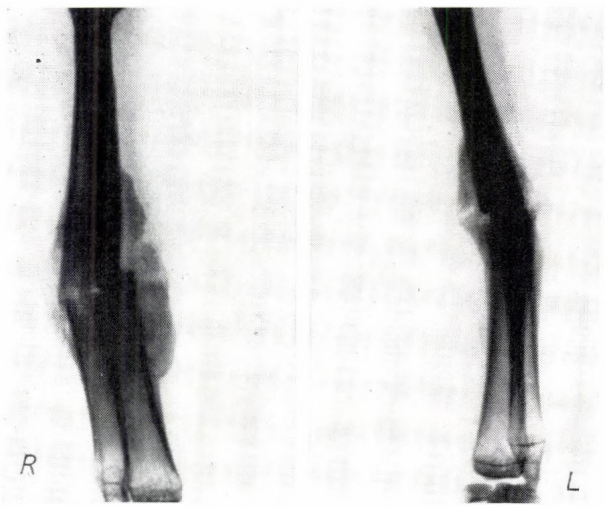

FIG. 8. Radiogram of a rabbit treated in a biological cell system. In the right fore-limb, abundant callus formation at the site of fracture. In the left fore-limb, a rather weak development of the callus

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FIG. 9. Histological picture of the site of fracture of the rabbit's right limb shown in Fig. 8 R. Abundant mineralized callus showing irregular structure surrounds the area of the fracture. A few cartilaginous foci lie outside the area of the callus proper at the level of the bone stump

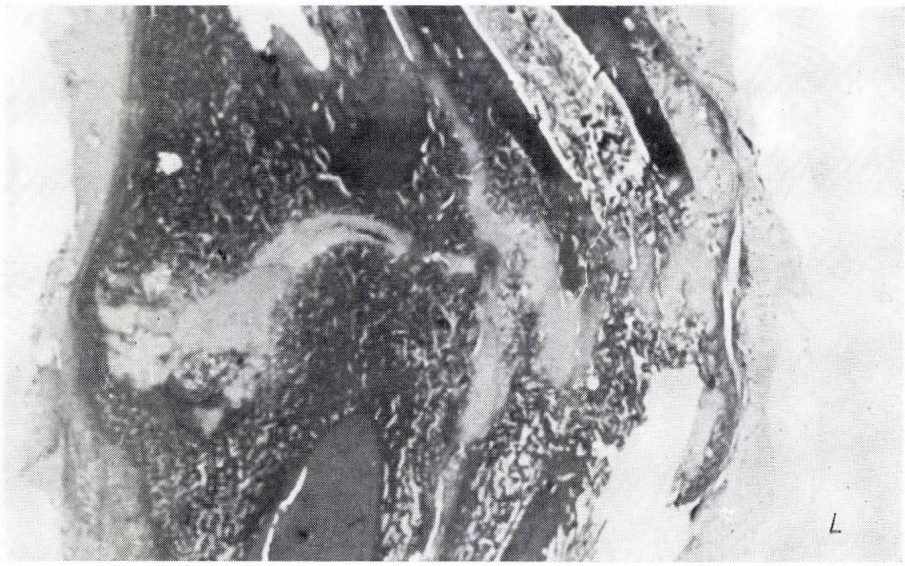

FIG. 10. Histological picture of the site of fracture of the left forelimb of the rabbit (Fig. 8 L). The entire width of the fracture is traversed by a strand of cartilaginous tissue with uneven projecting margins. Mineralized callus of irregular structure on both sides of the cartilaginous tissue. Picture of delayed bone concretion

Symp. Biol. Hung., 7, pp. 275—300 (1967)

LOCAL TISSUE METABOLISM AND THE QUALITY OF THE CALLUS

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BONE formation is known to occur in different ways. Bone may develop directly *(primary angiogenic* bone formation) and by preformative cartilaginous *(enchondral)* or connective tissular *(desmal)* ossification (Krompecher 1937, 1962). These three types of bone formation were recognized and verified in embryonic development as well as in regeneration (Oberdahlhoff 1948, Hasche-Klünder 1952, Krompecher 1962).

The question arises why bone forms in different ways. In the present work we wish to discuss whether any connection can be found between the way of bone formation and local metabolic conditions. The question seems to deserve an examination from various angles.

PHYLOGENETIC ASPECTS OF BONE FORMATION

First we should have a look at the phylogenesis of bone and that of supporting tissues in general, with special regard to the possible connection between the general and local metabolism of the animal organism and the quality of the tissues developed there.

On studying the phylogenesis of supporting tissues, gelatinous tissues with mucous ground substance are found in the low stage of development. The higher stages of development are characterized by the gradual appearance of the chorda dorsalis, chordoid and chondroid tissue and that of cartilage. The organisms of primitive animals consist of such tissues displaying a primitive metabolic activity. Blood circulation, existing in higher animals, is absent in such lowly creatures. Bone tissue appears in a higher stage of development since bone formation requires the presence of vessels. Bone having high metabolic requirements appears only in animals of higher phylogenetic development. The formation, development and maintenance of bone require *oxybiotic* metabolism and, consequently, the presence of blood vessels. The existence of this correlation, i.e. the interdependence of *vessels*, oxybiotic metabolism and bone, have been confirmed by examinations carried out in the field of phylogenesis, ontogenesis and regeneration.

Animals standing at lower levels of phylogeny, i.e. the snail, medusa and mussel, belonging to the group of molluscs, have no bones and are very poorly vascularized.

A more developed vascular system appears first with the chordates. According to Haller (1904), the Amphioxus, though having a vascular system,

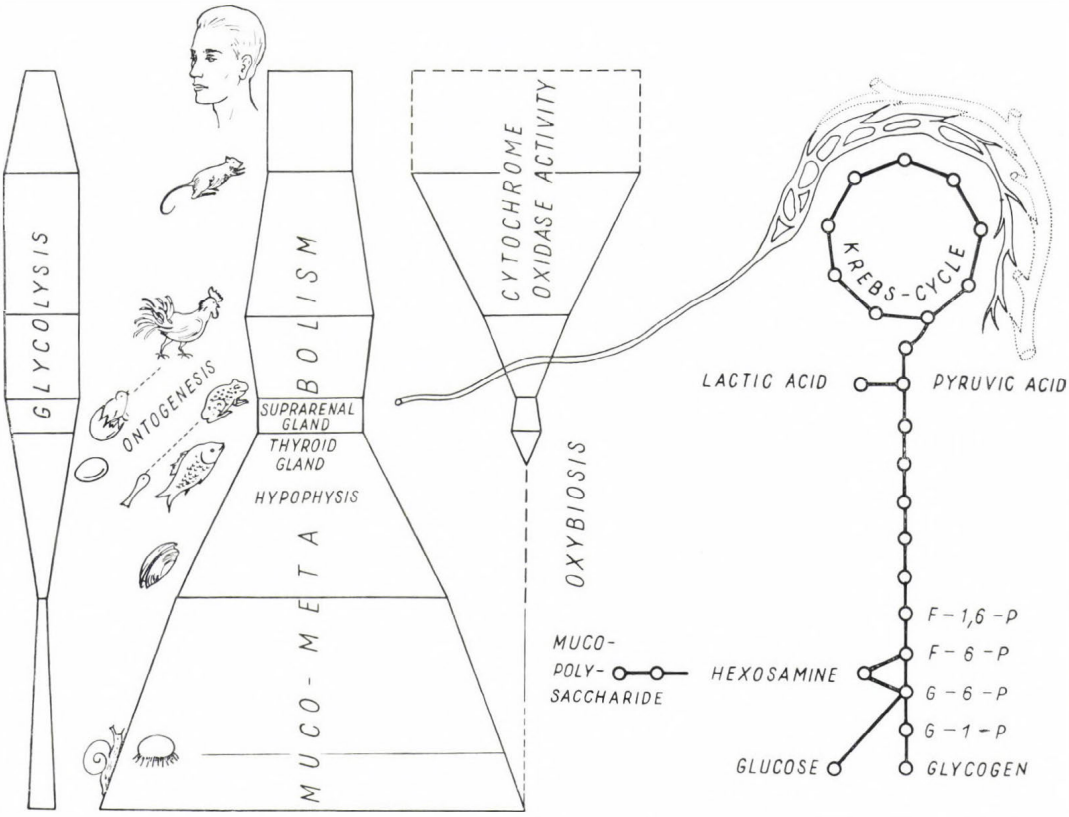

FIG. 1. Scheme of phylogenesis in the light of carbohydrate metabolism. In lower animals, mucopolysaccharide formation is predominant. Proceeding upwards in the phylogenetic line it decreases in importance, becoming narrower. Terminal oxidation prevails only in higher beings — as determined by cytochrome oxidase activity — i.e. where adequate blood circulation, red bone marrow, lungs and hormonal prerequisites are ensured. Fermentation (glycolysis) has an intermediary role

is heartless. Neal and Rand (1948) confirmed this finding. Thus, no marked oxybiotic metabolism can be present in the chordates. The first animal having a heart and blood circulation is the fish, in which a bone-like tissue appears devoid, however, of internal blood supply and red bone marrow. In the group of the amphibians, following the fish in the phylogenetical line, the Urodeles have no red bone marrow, whereas in the Anura there is an ingrowth of vessels into the bone and appearance of bone-forming red marrow. At the same time they have lungs as well. In this way, the prerequisite conditions of oxybiotic metabolism are given.

Returning to the molluscs, let us examine their metabolism, i.e. their biochemical composition from a metabolic viewpoint. Molluscs are built up of mucous, jelly-like tissues having a high mucopolysaccharide content

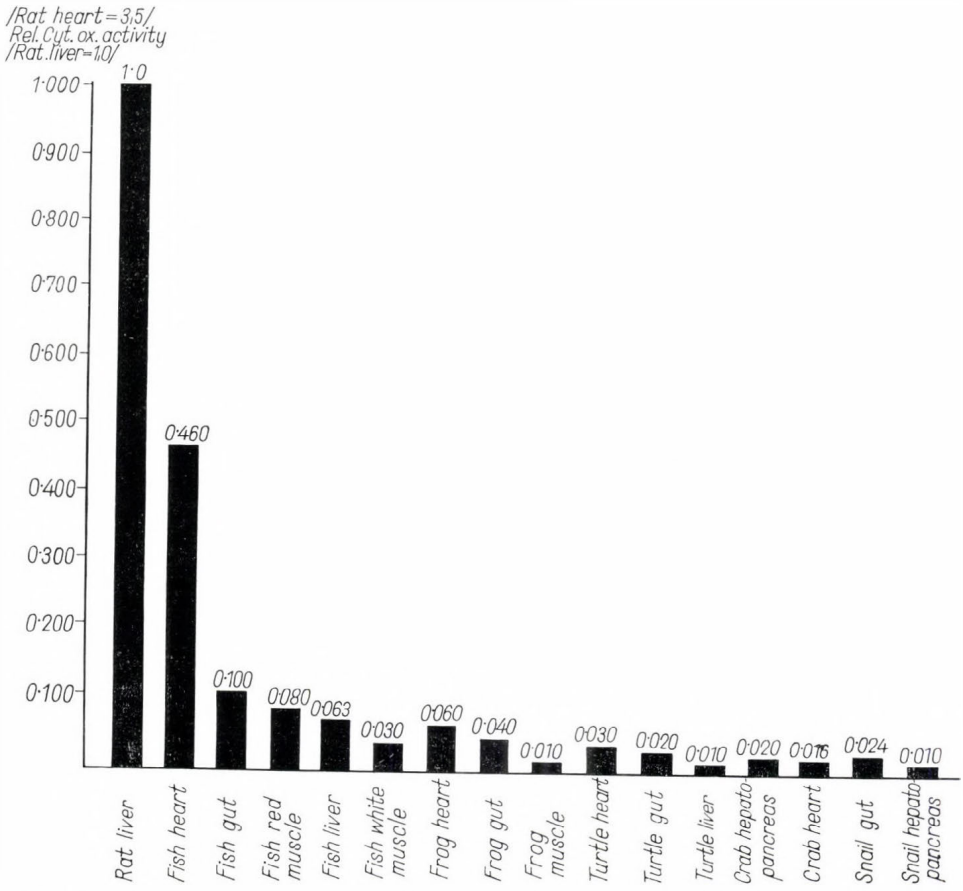

FIG. 2. Cytochrome oxidase activity of lower animals is extremely low, being about 1% of the values measured in rat liver employed as a basis for comparison (Krompecher, Ladányi and László 1966)

(Krompecher 1964, Krompecher et al. 1966). At the same time, the cytochrome oxidase activity of such tissues is very low, even scarcely demonstrable (Figs 1 and 2), differing in order of magnitude from the cytochrome oxidase activity of higher animals. The lactic acid content of the molluscs is likewise low. Ascending on the phylogenetic line, the mucopolysaccharide content of the animals is found to decrease (see the relative parameters in Fig. 1) with the simultaneous increase of cytochrome oxidase activity. Let us examine in which stage of development endocrine glands appear. This point seems to be of interest since the endocrine glands are known to have an effect on metabolism. In the course of phylogenetic development, the hypophysis appears first, followed by the *thyroid gland*. It should be mentioned that the thyroid gland is able to depress the mucopolysaccharide

level (Krompecher et al. 1961a, b, 1962) and at the same time enhances the oxybiotic processes. This is also observable in fish. In amphibians, the complete *adrenal gland* appears consisting of cortical and medullary elements lying in close proximity to one another (Berkelbach v.d. Sprenkel 1934). The frog, after the tadpole stage, has such a complete adrenal gland (Neal and Rand 1948). The presence of this adrenal gland — as it will be demonstrated later with experiments on animals — seems to play an important role in the increased growth of capillaries and in their penetration into other tissues, e.g. in bone. Phylogenetically, the red bone marrow in the frog appears here, too (Weidenreich 1933). Capillarization, as it is known, is a prerequisite condition of oxybiotic metabolism.

Comparing higher vertebrates than the amphibians with molluscs, concerning their biology and metabolism, it has to be noted that the conditions for an oxybiotic metabolism are not given in the molluscs since the adequate blood circulation required for oxybiotic metabolism is not ensured by the few capillaries present in such animals, to say nothing of the lack of oxyhaemoglobin and of cytochrome oxidase activity. Better conditions for oxybiotic metabolism begin with the amphibians. Ascending still higher up on the phylogenetic line, we can see the gradual development of a better capillary circulation with the parallel increase of cytochrome oxidase activity which is the essential condition of oxybiosis. At the same time, the mucopolysaccharide content decreases. Cytochrome oxidase activity and mucopolysaccharide content are inversely proportional to each other. As has been demonstrated by Oláh and Allemand and Krompecher et al. on some lower animals, the cytochrome oxidase activity of such animals is lower with an order of magnitude than that of higher animals (Fig. 2), displaying at the same time a high mucopolysaccharide level (Krompecher et al. 1966).

ONTOGENETIC ASPECTS OF BONE FORMATION

It is a generally accepted rule in biology that ontogenesis — at least in broad outlines — repeats phylogenesis. By studying the ontogenetic development of the bone system we find that in the course of the embryonic development of our skeletal system several steps of phylogenesis are repeated. In the development of the vertebral column the chorda dorsalis and cartilaginous preformation as cartilaginous vertebral column appear in subsequent stages succeeded later by the bony spine. In the course of the development of the extremities we can find the scleroblastema formed by closely packed mesenchyma bringing about the cartilage preformation in the axis, and the connective tissular (desmal) preformation in the perichondrial layer. It may, therefore, be stated that in the development of the skeleton, bone tissue, having high metabolic requirements (oxybiosis), is preceded mainly by cartilage tissue which has a more primitive metabolism, high mucopolysaccharide content and minimal cytochrome oxidase activity.

In addition to phylogenetic and ontogenetic examinations, it seemed to be instructive to perform comparative studies on different tissues of the adult organism as regards their metabolism.

DATA ON TISSULAR METABOLISM

There are certain tissues in the organism of higher animals which are not supplied by capillaries, e.g. cartilage, cornea, etc. In such tissues, high mucopolysaccharide content and extremely low cytochrome oxidase activity were also demonstrated. To discover what these observations mean as regards carbohydrate metabolism, we must turn back to events occurring on the biochemical level.

Three types of metabolism have been recognized in the tissues. According to the tricarboxylic acid (Szentgyörgyi—Krebs) cycle, glucose and glycogen are broken down through glucose-1-phosphate, glucose-6-phosphate, fructose--6-phosphate, fructose-1,6-diphosphate and subsequent stages, beyond pyruvic acid, entering the process of terminal oxidation. This scheme is well known to researchers, but the finding that the oxybiotic activity mentioned above is bound to the presence of vessels is less known (see Fig. 3). Under biological conditions, no process of oxidation can take place in the absence of capillaries. If a tissue is not supplied by a rich capillary network, ensuring the circulation of oxygenated blood, the breakdown of glucose and glycogen to CO_2 and H_2O – bound to high energy production — cannot occur according to the scheme mentioned above. In case of hypoxia, the breakdown can go as far as pyruvic acid and hence, it proceeds by fermentation to lactic acid, producing less energy. If not only hypoxia but a state of anoxia ensues in the tissues, the breakdown, in its majority, does not get to pyruvic acid, but it ramifies already at glucose-6-phosphate or fructose-6-phosphate and breaks down to mucopolysaccharides. The question of energy production of this metabolic process is still to be clarified. Thus,

(a) in *euxia*, the oxidation of glycogen occurs according to the Szentgyörgyi—Krebs cycle;
(b) in *hypoxia*, fermentation with lactic acid production prevails;
(c) in *severe hypoxia (= anoxia)*, mucopolysaccharides are formed.

These metabolic processes do not, however, occur independently of each other nor can one take place without the other, but they are generally present concomitantly in the same tissue. Still, if the tissue is supplied with *oxygenated blood*, *oxybiosis* will prevail. In case of hypoxia, fermentation with lactic acid will come to the foreground, but at the same time a certain degree of oxybiosis is still present, and an increasing mucopolysaccharidic type of metabolism. In a state of anoxia, oxybiosis is absent, fermentation decreases and *mucopolysaccharide metabolism* becomes predominant (Fig. 3). This latter type of metabolism is thus prevalent in avascular tissues (Castellani and Zambotti 1956). As regards the biochemical analysis of this question, I wish to refer to the excellent work of Zambotti and Bolognani (see p. 5).

From the foregoing the presence or absence of capillary supply seems to have a decisive importance in the metabolism of tissues.

Let us compare the metabolic conditions of some vascularized and avascular tissues. The tissues having a good capillary supply, e.g. liver, skin, brain and muscle, have a low mucopolysaccharide content ranging between

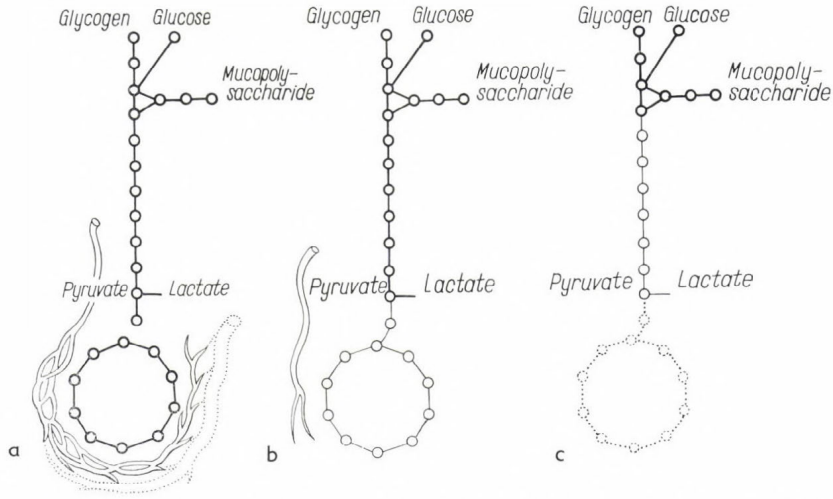

FIG. 3. Scheme of glucose and glycogen breakdown represented in relation to vascularization. a) Good capillary system and blood supply, high cytochrome oxidase activity. In such states of *euoxia*, *oxybiotic metabolism* prevails. b) In *hypoxia*, *fermentative metabolism* with production of lactic acid is predominant, and c) *anoxia* is characterized by *mucopolysaccharide* formation

157 and 531 mg%. In avascular tissues like cartilage, cornea, Wharton's jelly or vitreous body, the mucopolysaccharide content is very high: 1000 to 3000 mg%. These findings are concordant with our phylogenetic observations, according to which avascular tissues have very high mucopolysaccharide content. On the other hand, in the same tissues (cartilage, cornea, Wharton's jelly, vitreous body), the cytochrome oxidase activity was very low, sometimes even undemonstrable, precisely as it was found in avascular tissues of animals at lower stages of phylogenetic development, namely: high mucopolysaccharide content and very low cytochrome oxidase activity.

EXPERIMENTAL DATA

In addition to phylogenetic, ontogenetic and biological examinations, we wish to discuss the correlation between the extent of capillarization (euxia, hypoxia, anoxia) and tissular metabolism (oxybiosis, glycolysis, mucopolysaccharide metabolism) on the basis of experiments on regeneration and neodifferentiation carried out on an experimental model. Semiarthroplasty was performed on the knee-joint of dogs. The cartilaginous surface of the distal part of the femur was completely removed and, subsequently, a new articular surface was formed (Krompecher 1956, 1958a and see p. 383 as well as Fig. 7 on p. 288). The granulation tissue originating from the spongious bone gradually covered the new articular surface. From the fifth postoperative day adequate treatment was performed by moving the newly formed joints. By this procedure an increasing pressure

stress was exerted on the granulation tissue, in which the flexor and extensor muscles were more and more involved (Krompecher 1956, 1958a, Hadházy and Oláh 1958, Hadházy et al. 1959, 1961, 1962, Hadházy and Perjés 1962, Oláh et al. 1959, 1961, 1965, Oláh and Hadházy 1962, Krompecher and Tóth 1964, 1965).

As it was demonstrated in this experiment, the capillaries of the granulation tissue became gradually occluded as a result of compression forces (the initial 6% of the territory occupied by capillaries diminished to 1·3% in 70 days, then it fell to 0). The haemoglobin content of the regenerating articular surface — expressed in Fe^{+++}/g tissue decreased from 40·7 to 13·1 γ, then to nil. Consequently, parallel to capillary pauperization, oxybiotic metabolism was replaced by anaerobic glycolytic metabolism. This metabolic shift was verified by a considerable increase in lactic acid production and content observed in the period between the 20th and 23rd days (Hadházy and Oláh 1958, Hadházy et al. 1962, Hadházy and Perjés 1962, Oláh et al. 1959, 1961, 1965, Oláh and Hadházy 1962). When the granulation tissue displays complete vascular pauperization, marked increase of mucopolysaccharides occur in the tissue and the granulation tissue differentiates to cartilage. At sites where the injured bone trabeculae are surrounded by granulation tissue rich in capillaries, and functional stimuli are ensured, direct angiogenic bone building is continued. However, at sites where, owing to the effect of compression, the vessels become compressed, a subsequent state of hypoxia ensues. Consequently, local metabolism undergoes a shift in the direction of mucopolysaccharide type of metabolism, and corresponding to it an avascular tissue having a a mucopolysaccharidic metabolism, namely, cartilage tissue is formed. This process has been reproduced and followed step by step in more than 500 cases in experiments on dogs, carried out with my co-workers and using a great variety of methods (Hadházy et al. 1963). The discussion on the effect of pressure stresses resulting in formation of cartilaginous callus (Krompecher 1937, 1956) was unequivocally settled by the experimental results of Yamagishi and Yoshimura (1955) obtained on 250 rabbits. Concerning the biophysical demonstration of vascular compression, reference is made to the work of Krompecher and Tóth (1964, 1965).

In a series of experiments in progress we have made attempts

1. to accelerate the rate of capillary reduction;
2. to maintain a state of adequate vascular supply;
3. to increase vascularization in granulation tissue.

According to these experiments, in case of good vascularization maintained or increased by acetylcholine administration (see p. 361) no cartilage differentiation occurs, while decreased vascular supply, due to noradrenaline administration, brings about an early cartilage formation or the appearance of a bradytrophic tissue similar to it (Kostenszky 1964).

Of the results of our investigations concerning the experimental formation of articular cartilage, let us turn our attention to the consistent finding that after some weeks, parallel to vascular pauperization, a decrease

in lactic acid production and content and, simultaneously, an abrupt rise in the mucopolysaccharide (hexosamine) content of the tissue were noted.

During the process of cartilage differentiation, the granulation tissue exhibits three characteristic stages (maxima):

1. Richly vascularized tissue displaying numerous cross-sections of vessels having a high haemoglobin content (conditions of oxybiosis);
2. Fibrous tissue with few capillary cross-sections, low haemoglobin content but high lactic acid content (characteristics of glycolysis);
3. Cartilage tissue with minimal number of capillary cross-sections, very low haemoglobin content, considerable decrease of lactic acid content showing at the same time a high mucopolysaccharide content; in the given case: chondroitin sulphate (characteristics of mucopolysaccharide metabolism). For a detailed description see Hadházy et al. (1963) and p. 75 in this volume.

Thus, by the experimental reduction of the vascular supply, we have been able to change the local metabolic circumstances. Under these changed conditions, undifferentiated cells may differentiate to a tissue corresponding to the given primitive metabolic possibilites (in this case to cartilage), i.e. to a tissue which is devoid of capillaries and haemoglobin, which has a minimal cytochrome oxidase activity or none at all, its lactic acid production and content is likewise low, while its mucopolysaccharide content, measured in hexosamine, is very high.

*

The question arose whether this shift to mucopolysaccharide type of metabolism does require a period of several weeks, and whether the intermediate stage of glycolysis between the stages of oxybiosis and mucopolysaccharide metabolism is indispensable in this shifting process. Another question was whether this metabolic shift may be observed in other tissues as well.

Experiments were conducted on guinea-pigs and rats, in which the back skin of the animals (an area about 25 cm^2) was subjected to freezing with dry ice. While prior to freezing the tissues of the cutis were richly supplied by capillaries, freezing with dry ice applied for two minutes resulted in destruction of the capillary network as evidenced by morphological examinations performed 30 minutes later. By histochemical examinations it was demonstrated that even 30 minutes after freezing mucopolysaccharides appear in the frozen skin of the guinea-pigs, which were fairly demonstrable, especially on the third and fifth day after intervention. At the same time, the biochemical examination of the frozen skin piece disclosed that after 30 minutes the content of hexosamine increased by about 15% and after three and five days this increase amounted to 30 to 40%. After this date the hexosamine content was found to diminish slowly parallel with the healing of the frozen skin. At the same time, lactic acid was found to diminish by about 10% in the first 30 minutes and by about 30% after 3 to 5

days. After this date the lactic acid content was likewise found to return slowly to the original value (László 1966).

Thus, by these examinations it was demonstrated that metabolism may shift within a short time in other tissues, too, e.g. moderately frozen skin tissue did not die but its former predominantly oxybiotic metabolism changed to a much more primitive mucopolysaccharide type of metabolism, passing over the metabolic phase of glycolysis. By comparing the curve representing the metabolic changes of the frozen skin with that of cartilage formation, remarkable similarities may be observed: capillary occlusion —occurring in cartilage formation after about 33 days and in case of freezing shortly after injury—brings about in both tissues a steep increase of hexosamine content and a simultaneous steep decrease of lactic acid content. It may be added that after healing of the frozen skin, parallel to re-growth of capillaries, the curves—showing a reversed image—return to their former normal level.

This investigation has added support to our earlier statement that if local hypoxia or anoxia ensues in the tissue, without the immediate destruction of the whole tissue, the *metabolism of the tissue may shift* from its formerly predominant oxybiosis to mucopolysaccharide type of metabolism. The latter metabolism is characterized, as mentioned before, by the absence of capillaries and haemoglobin, minimal or no cytochrome oxidase activity (i.e. deficient oxybiosis), as well as by a considerable decrease in the locally produced lactic acid, showing at the same time a marked increase in local mucopolysaccharide production and content. These data indicate that if capillaries are absent, glucose and glycogen are shortly broken down to mucopolysaccharides (see Fig. 3). Simultaneously, fermentative metabolism (glycolysis) becomes inhibited and oxybiosis is reduced to minimum.

This statement has been substantiated by phylogenetic and ontogenetic findings as mentioned previously, comparative examinations on vascularized and non-vascularized tissues, and by extensive experimental series on the neodifferentiation of cartilage, as well as by metabolic shift observed in the course of experiments on frozen skin.

PATHOLOGICAL REFERENCES

The statement that the metabolism of a tissue *in situ* is dependent on its vascular supply, has been confirmed under various pathological conditions, namely, in C_3H mouse cancer (Krompecher and Berencsi 1960) in tuberculosis (Berencsi 1961, Krompecher 1960, 1964), in varicose ulcer (Krompecher and Szodoray 1964), in rheumatism (Krompecher 1960), in silicosis (Kardos et al. 1961), in thermal injuries (Berencsi and Krompecher 1963, László 1966), as well as in experiments on tissue strangulation (Schmidt et al. 1963). These results indicate that a similar mechanism works under all these circumstances.

GENERAL BIOLOGICAL CONCLUSIONS

Concordant phylogenetic and ontogenetic data, as well as consistent findings obtained under biological, regenerative and pathological circumstances indicate that tissue metabolism—depending on vascularization—follows a general biological rule:

(a) in euxia (i.e. good capillary supply) oxybiosis is predominant;
(b) in hypoxia (reduced capillarization) anaerobic glycolytic metabolism prevails;
(c) in anoxia (absence of capillaries) mucopolysaccharide type of metabolism sets in, provided the local injury is not so severe as to involve the immediate destruction of the whole tissue.

It has been recognized that there are tissues requiring good vascular supply, while others, the so-called bradytrophic tissues, do not require any vascularization.

It has also been concluded that by changing the degree of vascularization of a tissue, its metabolism could be modified, which modification then leads to transformation of the structure of the tissue. Tissues in a stage of regeneration (i.e. in a plastic state) were found to be particularly suitable for such experiments.

METABOLIC CONDITIONS IN REGENERATIVE BONE DEVELOPMENT

Let us examine whether and to what extent the biological rules mentioned above, can be applied to bone, in particular to regenerating bone.

It should be borne in mind that under normal circumstances bone is in a constant slow reconstruction. There are layers of reserve cells on all bone surfaces, most markedly in the cancellous bone tissue. The periosteum is likewise provided with reserve cells. The presence of a capillary network is indispensable for bone.

Under regenerative conditions, the *vascular supply* of bone may be good, mediocre or even insufficient. In *fractures of spongious bone* good vascularization often persists in areas bordering the broken bone ends. The surfaces of the fractured spongious bone frequently become adjusted and the fractured ends remain fixed to each other. In such cases the fracture line is not exposed to mechanical stress and so the vessels can grow into the relatively narrow gap between the ends anastomosing there, and primary angiogenic bone formation starts.

The newly formed bone trabeculae become anchored by appositional growth in both fractured bone fragments, connecting the fractured ends by a trabecular lattice (Fig. 4). The close connection between the newly formed bone and vessels is readily recognized in pictures demonstrating primary angiogenic callus formation in cancellous bone (Fig. 5). Wide, sinuosoid capillaries (with a diameter of about 8 to 10 red blood cells one beside the other), perivascular tissue, osteoblasts and formation of bone trabeculae mark the process of angiogenic bone development. Soon the primary reticulum of the red bone marrow develops from the perivascular

FIG. 4. In healing of fractures of spongious bone, good vascular supply and, consequently, primary angiogenic callus formation occurs

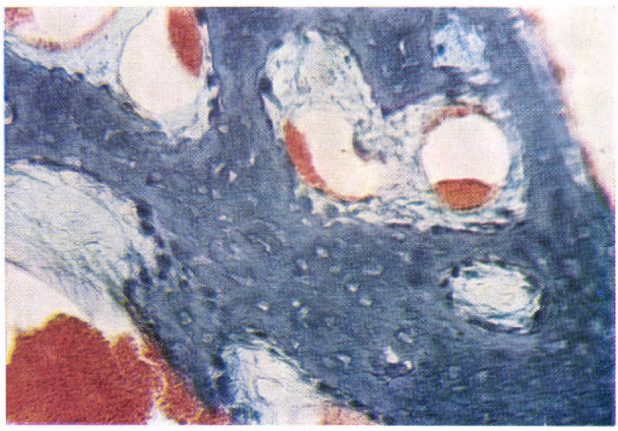

FIG. 5. Picture representing a portion of a primary angiogenic callus. Bone trabeculae are formed around the vessels

tissue, followed by the formation of haematopoietic red bone marrow. Their new formation is clearly shown by the topography and staining of the bone trabeculae (Fig. 5), and from the time elapsed between the date of fracture and that of histological elaboration conclusions may be drawn regarding the rapid rate of their ossification. The newly formed bone trabeculae can be unequivocally distinguished from the old ones even at the sites where new trabeculae have formed by appositional growth on the old ones. On the margins of the bone trabeculae, osteoblasts are present sometimes scattered, sometimes in close proximity to each other.

By the correlation of the developing bone to rich capillarization, the conditions of oxybiotic metabolism are ensured. In other words, where there is a good capillary supply between two fractured bone surfaces, *primary angiogenic bone formation* occurs without preformation of cartilage

or connective tissue. As regards causal investigations on bone development, it should be borne in mind that in these experiments—supposing a healthy organism—we have to deal with a process taking place between broken bone surfaces and not with a process occurring at any other site of the organism where there is no bone in the vicinity.

As it will be described later, direct bone formation does not take place in all cases but only where local blood supply is undisturbedly ensured between the fractured bone surfaces.

In *fractures of compact bone* the situation is quite different whether the blood supply of the broken bone ends, or whether the vascularization of the interfragmental space is concerned. As a result of the injured capillaries of the Haversian systems, the metabolism of bone areas in the vicinity of the fracture becomes seriously disturbed causing necrosis of varied extent in these territories. In contrast to the good blood supply of the cancellous bone, the compact lamellar bone and the Haversian systems themselves have a poor vascular supply. The interfragmentary space is thus, — at least for a time — devoid of any noteworthy blood supply coming from the compact bone surfaces. A new blood supply can develop partly, from the periosteum and partly from the medullary cavity. We shall now examine whether or not the prerequisites of the formation of a capillary network are given in the interfragmentary space, and if such conditions are absent which are the causes and results of their absence.

HEALING OF FRACTURES OF COMPACT BONE IN CASE OF COMPRESSION TREATMENT

The broken compact bone fragments, whether by synergistic activity of the flexor-extensor muscles or by some loading of the limb, collide and are pressed to each other so much that the vessels at the site of fracture become compressed or even torn. The torn vessels bleed into, and about, the fracture area and this results in a blood clot filling up the narrow gap between the fragments, and serves as a nutrient medium for the granulation tissue developing there. The granulation tissue cells creep into the gap between the fragments, they multiply there and bring about the callus connecting the fractured ends. The further differentiation of these granulation tissue cells is highly dependent on metabolic conditions present there. In the absence of vessels, the nutrition and oxygen supply of the granulation tissue decrease so much that the cells become incapable of oxybiotic metabolism. The rate of glycolysis diminishes as well. The existence of the cells is, however, ensured even under such adverse circumstances by a mucopolysaccharide-type of metabolism. In this environment the cells of the granulation tissue, while producing cartilage ground substance with a high mucopolysaccharide content, differentiate to chondrocytes. The prominent feature of the cartilaginous callus formed under such circumstances is avascularity (Fig. 6). The cartilaginous callus formed in such a milieu has certain valuable properties, namely, it provides a firm connection between the broken fragments and it can support loading (compression). On the periosteal margins — due to the presence of certain wobbling movements — the cartilaginous callus is delimited by a layer of fibrous connective tissue, by which the

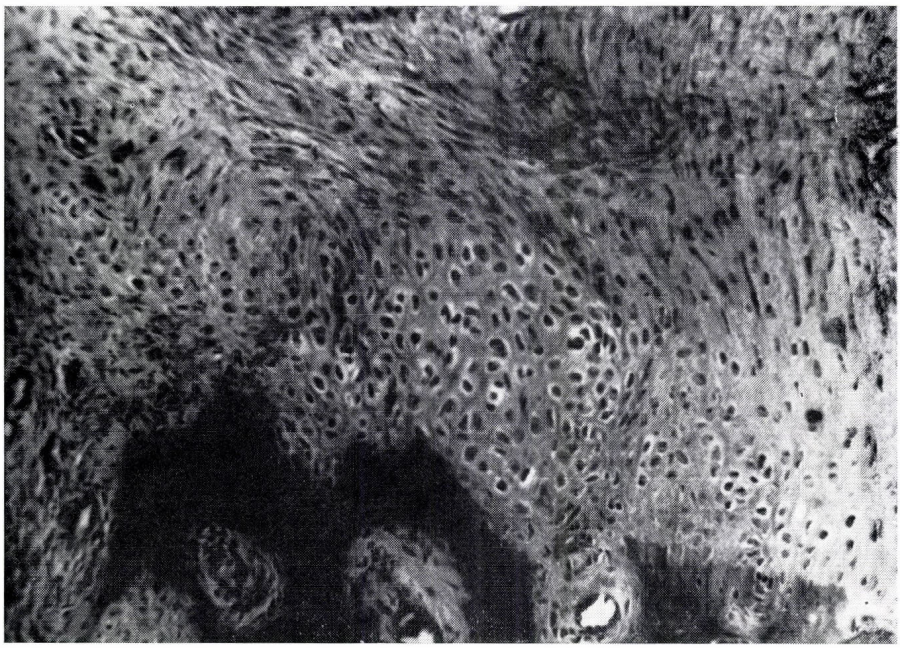

FIG. 6. Cartilaginous callus formed between the fractured bone ends as a result of postoperative treatment (compression)

tensile strength between the fracture ends is also ensured. Nevertheless, the most valuable feature of the cartilaginous callus is its capacity of joining the broken fragments firmly, by which capillary ingrowth and resultant cartilage resorption and simultaneous building of new spongious bone can take place. The formation of new cancellous bone occurs in a similar way as in enchondral ossification, i.e. advancing from the metaphysis towards the epiphyseal cartilage. But while in enchondral bone formation the epiphyseal cartilage ossifies only on the metaphyseal side, and the cartilage disk grows on the epiphyseal side, in the formation of cartilaginous callus the replacement of the cartilaginous plate by bone occurs from both sides. The cause of this difference is not yet known.

Although the cartilaginous callus is devoid of vessels, it can give place, in a few weeks or months, to capillaries invading from the broken fragments. Subsequently, the cartilaginous callus becomes resorbed and replaced by cancellous bone rich in capillaries. Thus, the transient cartilaginous callus having a hypoxic metabolism is replaced by cancellous bone having an euoxic metabolism.

HEALING OF FRACTURES OF COMPACT BONE IN CASE OF TRACTION TREATMENT

If traction is applied on fractured bone ends, a large gap arises between the fragments which will soon be filled up with blood originating from the

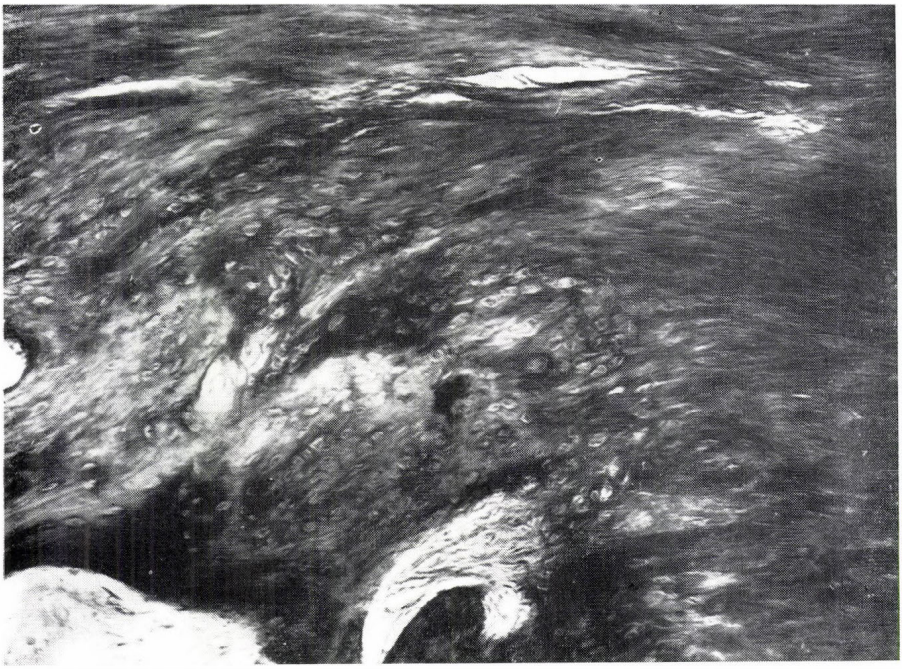

FIG. 7. Connective tissue callus formed between the fractured bone ends as a result of postoperative treatment (traction)

ruptured vessels forming a blood clot of a considerable size. Next, the blood clot becomes organized and granulation tissue cells followed by vessels penetrate into it. Prolonged traction determines the shape and direction of the differentiation of the granulation tissue cells. The cells become spindle-shaped. In this way, as a result of traction, a desmal callus develops corresponding to the tension forces acting there (Fig. 7). In this tissue exposed to tension there are but few vessels.

By comparing the vascularization of the *desmal* (connective tissue), *primary angiogenic* and *enchondral* (cartilaginous) calluses, it can be stated that while enchondral callus is devoid of capillaries and the primary angiogenic callus is richly supplied with wide sinusoid vessels, the vascularization of the desmal callus is rather poor, blood being scantily provided by a few, narrow vessels present among the stiff bundles of collagen fibres. The capillary network of desmally formed bone is generally poor, especially compared with that of primary angiogenic bone.

In the course of development of desmal callus, not all the cells of the granulation tissue take part in the formation of collagen fibres, but a part of them will transform to osteoblasts producing bone. The collagen fibres of the callus consisting of preformed connective tissue will be built in the newly formed bone. In this way, the new bone will contain, besides cyto-oste-

ons, large amounts of preformative connective tissue and a modest capillary network. According to experience, this type of bone is far from being permanent, its duration being rather limited, since the traction treatment applied in the first period after fracture is stopped after a few weeks and thenceforth this callus will be subjected chiefly to pressure stresses. Corresponding to the new mechanical demand, the bony callus undergoes a re-organization regarding both its structure and vascularization to meet the requirements of the new situation.

Pseudarthrosis, i.e. fibrocartilaginous callus (Krompecher 1956, Yamagishi and Yoshimura 1955) develops from the desmal callus as follows: owing to torsion of the collagen fibre bundles, the vessels become occluded and, consequently, the reserve cells, corresponding to the new metabolic (= severely hypoxic) environment, do not differentiate to osteoblasts but produce cartilage ground substance. In this way, the desmal callus transforms to *fibrocartilaginous callus*. Pseudarthrosis built up of fibrocartilage —since no capillary ingrowth occurs normally in fibrocartilage— has no inclination to healing. This problem has been extensively discussed by Yoshimura and Yamagishi at this Symposium.

HEALING OF FRACTURES OF COMPACT BONE BY STABLE OSTEOSYNTHESIS

In fractures of compact bone, direct bone union (i.e. by primary angiogenic callus) has been successfully achieved by stable osteosynthesis (Willenegger et al. 1962) without the intermediary stages of enchondral or desmal union of fragments. These experimental investigations and clinical results of Willenegger et al. (1962) are important both theoretically and clinically. Concerning earlier experimental work in this field, reference should be made to the work of Matzen (1954), notably, to Figs 2 and 3 of his work. In this respect, the question of the breaking strength of calluses formed by primary fracture healing is raised.

Thus it may be concluded that if primary angiogenic callus is formed — which is usually the case in fractures of cancellous bone—oxybiotic metabolism, due to good vascular supply, is continuously ensured.

In case of enchondral callus, the metabolism of the tissues decreases on account of the poor vascularization existing between the broken fragments pressing against each other to the level of mucopolysaccharide-type metabolism. This is the form of existence of cartilage rich in chondroitin sulphate. This metabolic state is, however, temporary, since after full development of the cartilaginous callus, its resorption will soon begin by vessels invading from both fractured ends, and this cartilaginous callus having a primitive metabolism will be replaced in a few weeks by richly vascularized cancellous bone having an oxybiotic metabolism.

Desmal callus is relatively poor in vessels, consequently, its metabolic conditions are also poor in comparison with those of primary angiogenic callus and its organization is delayed.

EXPERIMENTS ON BONE INDUCTION WITH SPECIAL REGARD TO THE ROLE OF VASCULARIZATION

As it has been shown in the foregoing, oxybiotic metabolism is dependent on the vascular supply of the tissues. This finding has been substantiated by phylogenetic data and experiments on chondrogenesis. We have seen that as a result of vascular pauperization the local tissue metabolism undergoes an adaptative shift from the former high oxybiotic metabolism to a more primitive type of metabolism characterized by high mucopolysaccharide content. This shift of metabolism (Hadházy et al. 1963) which has been repeatedly reproduced experimentally, proved to have a regressive character, namely, a higher form of metabolism—as a result of our intervention—has shifted to a primitive form of metabolic activity. The question arose whether we are able to induce the organism to transform a tissue having a low metabolism to a tissue with higher metabolic activity.

Starting from our finding that good vascular supply is essential for oxybiotic metabolism, it was obvious that the condition *sine qua non* of the transformation of the metabolism of a tissue is whether we can induce capillary ingrowth in that tissue. Many attempts have been made to induce the vessels of a living organism to grow into an avascular tissue. As it is known, the vessels stop on the border of the articular cartilage, cornea, etc. and do not penetrate into these tissues even in a lifetime. Still, the problem did not appear to be altogether hopeless, since in enchondral ossification the vessels are able to penetrate continuously into the epiphyseal cartilage, and capillaries have been demonstrated to grow into the cornea as well under certain pathological circumstances.

It seemed thus, reasonable to study the possibility of developing a reproducible method by which capillary ingrowth can be achieved.

After several years of unsuccessful experimentation, finally we succeeded in obtaining positive results (Krompecher and Kiss 1961, Kiss and Krompecher 1962).

The experiments were performed on pieces of human umbilical cord. As it is known, the umbilical cord (Wharton's jelly) has no vascular supply, it consists of primitive mucous tissue, high in mucopolysaccharide content (1750 mg%). Pieces of umbilical cord were explanted to chorioallantoic membrane of hen eggs incubated previously for 9 to 10 days.

While in cases of liver or blood clot explants the chorioallantoic vessels soon grew around the graft and penetrated into it, in cases of umbilical cord explants the vessels, though growing round the graft, never penetrated into them. Similarly, the vessels were unable to grow into cartilage explants.

After several years of experimentation, of the numerous substances tested by Krompecher and Kiss (1961) the saline extract of complete adrenal homogenates proved to be most effective in stimulating vascularization. If a piece of Wharton's jelly or cartilage had been pretreated with adrenal extract (Krompecher and Kiss 1961), prior to explantation to chorioallantoic membrane, the capillaries of the chorioallantoic membrane consistently grew into the graft. The extent of capillary ingrowth corresponded to the normal rate of vascularization, the planimetrically determined percentage

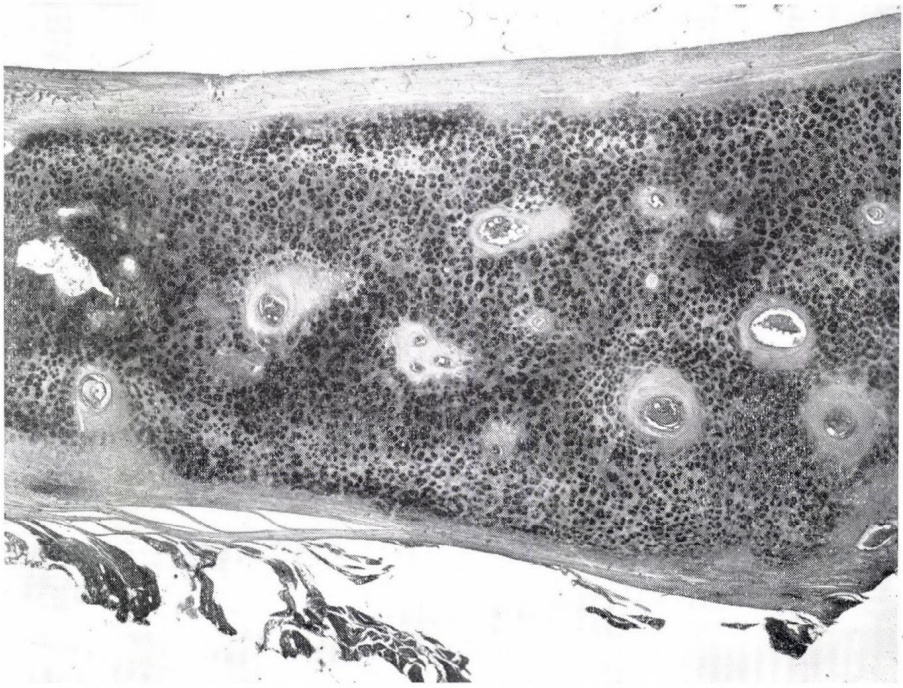

FIG. 8. Following injection of complete adrenal extract into the costal cartilage of the dog, capillary ingrowth and formation of perivascular bone tubules are noted (Kiss 1964)

ratio of the capillary cross-sections being about 4% compared with the surface of the whole explant.

This experimental material was, however, not appropriate for the study of tissular metabolic changes since, on account of the maturation of the incubated eggs, i.e. the hatching of chickens, the follow-up examination of these series of biological experiments was impossible.

The question arose whether or not, on the analogy of the explanted umbilical cord or cartilage, it is possible to induce capillary ingrowth in the living organism.

Experiments were conducted on dogs by puncturing about 20 holes with a sharp dental probe in the fourth, fifth and sixth costal cartilage of dogs (under ether anaesthesia). Physiological saline was injected into the holes of the fourth costal cartilage, and adrenal extract into the fifth and sixth ones. The corresponding portions of the heterolateral cartilages served as controls. The dogs were sacrificed after different intervals following intervention. No capillary ingrowth occurred in the costal cartilage in which physiological saline was injected or were left empty. On the contrary, gross anatomical and histological examinations disclosed that from the perichondrium capillaries had grown into the holes of costal cartilages in which adrenal

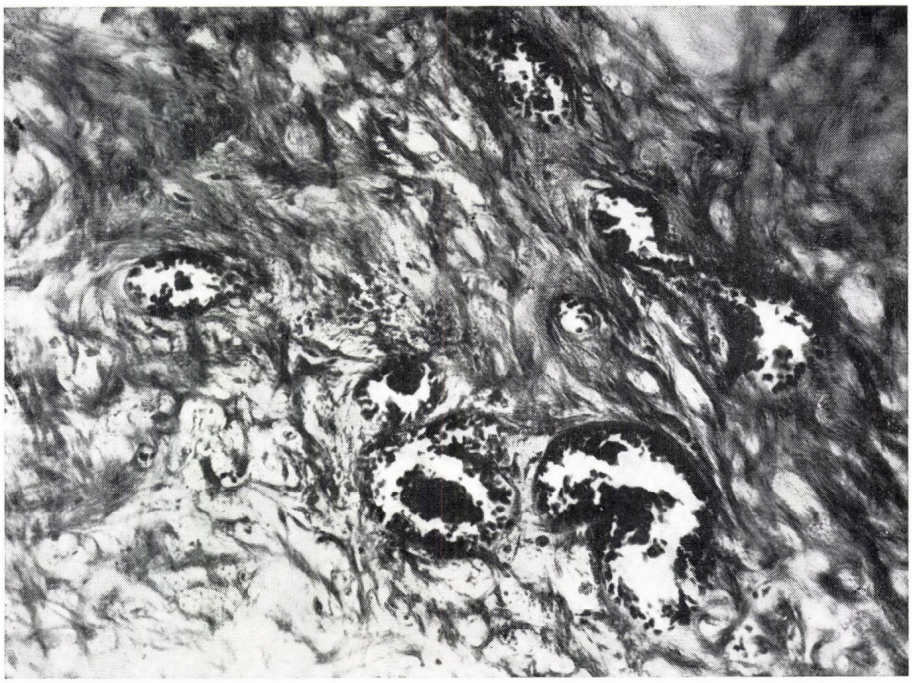

FIG. 9. On injecting adrenal extract into the intervertebral cartilage disk of the dog, capillary ingrowth occurs in the early stage (Krompecher and Kiss 1963)

extract was injected. Of special interest as regards this report was the observation that *the capillaries grown into the holes of the costal cartilage were surrounded by bony tubules* (Fig. 8). The difference between the controls and the costal cartilage in which adrenal extract was injected, resulting in ingrowth of vessels and bone formation, was evidenced radiographically as well (Kiss 1964).

The finding that if adrenal extract was injected into the costal cartilage, capillary ingrowth and resultant osteogenesis were consistently noted, prompted us to examine whether or not this process can take place in similar tissues localized elsewhere in the organism. Experiments were performed by injecting complete adrenal extract into the intervertebral cartilage of dogs through a canal bored in the vertebral body reaching the intervertebral disk (Krompecher and Kiss 1961, 1963). Two months after a single injection of adrenal extract, bone condensation and vascular ingrowth were noted on the margins of the vertebral body (Fig. 9), and six months later, the cartilage disk was interwoven with vessels and the former fibrous cartilage was replaced by cancellous bone. This richly vascularized young bone connecting the neighbouring vertebrae was fairly demonstrable radiographically, anatomically (Fig. 10), microscopically (Fig. 11) and in its development (Fig. 12).

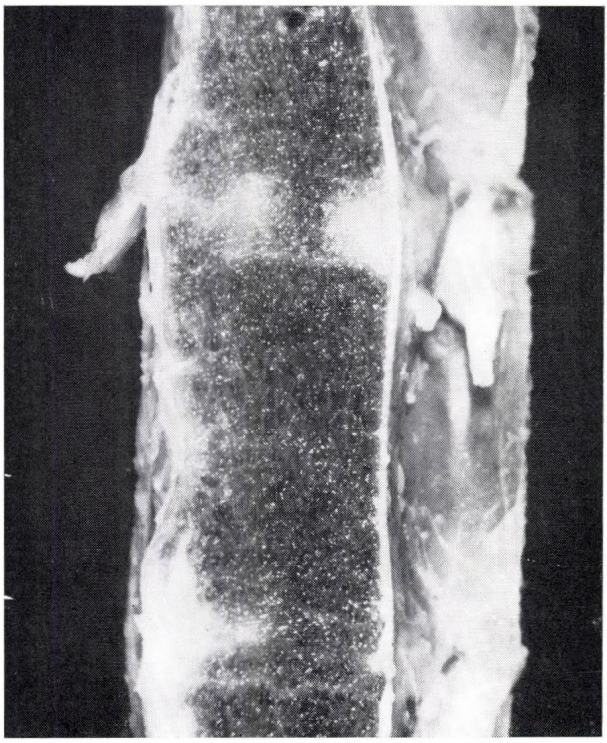

FIG. 10. Injecting adrenal extract into the intervertebral disk of the dog, ingrowth of vessels and subsequent bony unification of the neighbouring vertebrae are demonstrated. Gross anatomical specimen (Krompecher and Kiss 1963)

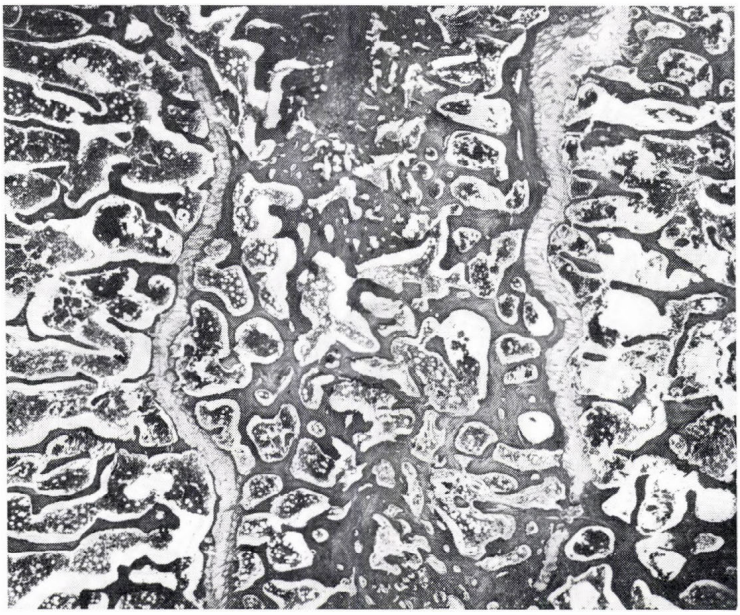

FIG. 11. Six month after injection of adrenal extract in the intervertebral cartilage disk of the dog, spongious bone has replaced the cartilage disk (middle part of picture). The newly formed bone contains haematopoietic red bone marrow (Krompecher and Kiss 1963)

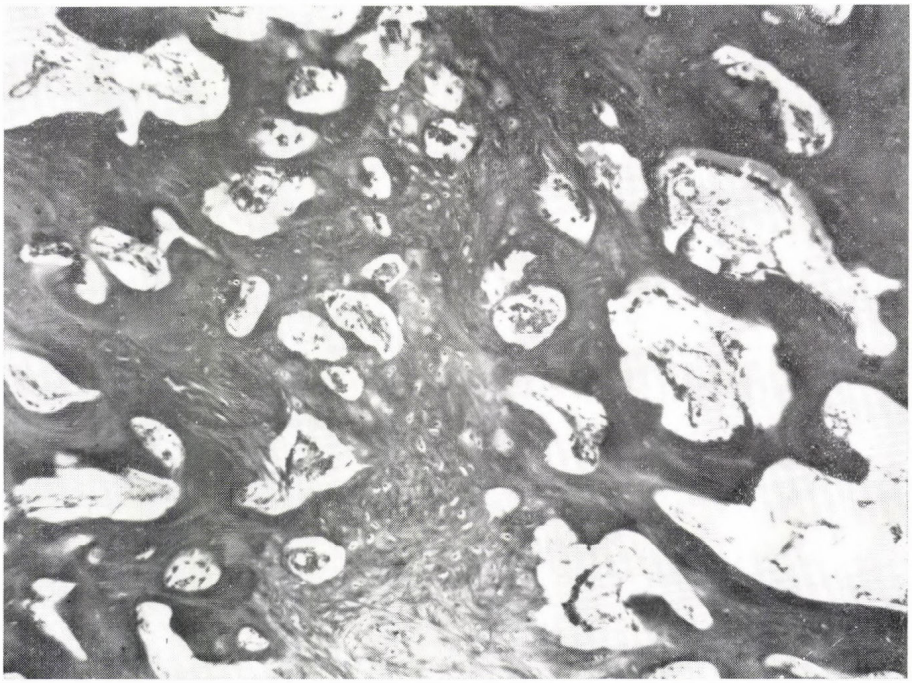

FIG. 12. Six months after injection of adrenal extract, bony unification of the neighbouring vertebrae. A remainder of the cartilage disk (lower middle part of picture) is still visible. Vessels are advancing from both sides and bone formation is in progress (Krompecher and Kiss 1963)

From this series of experiments the following conclusions can be drawn with regard to bone formation, vascularization and metabolism: at sites where a vessel grows into cartilage destroying it, bone is formed. The same was observed in ontogenesis, e.g. in enchondral bone formation and the same result was obtained in experiments on adult animals, i.e. if we succeeded in inducing capillary ingrowth in cartilage bone formation was obtained.

By the ingrowth of vessels into cartilage, the conditions of oxybiotic metabolism are ensured and, consequently, the cartilage having a primitive mucopolysaccharide metabolism, will be replaced by bone tissue with an oxybiotic metabolism. In the lacunae of this newly formed bone, haematopoiesis will soon start. This shift of metabolism and change of structure has a progressive character, i.e. here a lower form of metabolism has shifted—as a result of our intervention—to a higher form of metabolic activity. Thus, under adequate experimental circumstances we have been able to influence tissular structure and metabolism not only in a regressive, but also in a progressive direction.

The ingrowth of vessels, change of local tissue metabolism and appearance of new bone tissue are evidences of the *inseparable unity of form and function*

in life. The change of form has an effect on function, and function in its turn affects the development of form. Form and function are thus in a constant interaction.

As it has been described, we have succeeded in obtaining tissular differentiation by local administration of adrenal extract. Previously we have already pointed out the importance of the adrenal glands in phylogenesis; the appearance of the red bone marrow, pulmonary respiration, cortical grey matter, i.e. tissues or processes requiring oxybiotic metabolism are all connected with the appearance of the suprarenal glands.

The results of the experiments, described above, in which bone has formed as a result of adrenal extract administration, are in agreement with the relevant phylogenetic data.

EXPERIMENTAL RESULTS ON THE STIMULATION OF BONE REGENERATION

The question whether or not bone regeneration can be stimulated has been widely discussed in the literature.

As has been demonstrated, vascularization has an essential role in bone formation. Of the numerous conditions and causes of ossification the role of bone salts deposited in the bone ground substance is emphasized.

We shall now discuss whether or not we are able to stimulate bone formation by administration of adequate bone salts.

It has been previously reported that peroral administration of pulverized egg-shell is effective in the prophylaxis and healing of rickets by strengthening bone and stimulating, through the medication of the vascular system, the haematopoietic activity of the red bone marrow (osteohaematopoietic entity) (Krompecher 1957). It has been demonstrated that egg-shell consists of the same components and trace elements as bone, i.e. Al, Ag, Ba, C, Ca, Cd, Cl, Co, Cu, F, Fe, Mg, Mn, Mo, La, Na, Ni, P, Pb, S, Si, Sn, Sr, Ti, V, and Zn. In addition, egg-shell contains Vitamin D_3 which is known to be important in bone building, calcium soluble in lipids, citric acid and ooporphyrin (Krompecher 1958b).

In their experiments on rats, Lelkes and Mészáros (1960) found a more rapid fracture healing and a better callus if powdered egg-shell was perorally administered to the rats. As a result of these findings, Tarsoly (1963) carried out investigations on the effect of local administration of pulverized egg-shell in case of bone defects in dogs. A mixture of egg-shell and plaster (1 : 1) was prepared. Streptomycin and penicillin were added as well as physiological saline to make the mixture more plastic. Two cavities (10 to 15 mm dia) were chiselled in the tibia of dogs, and one of them was filled in with a mixture of egg-shell and plaster and the other one was left empty for control. As it is demonstrated on a 14-day old specimen, the mass of egg-shell and plaster has been broken down by giant cells of the granulation tissue in which wide capillaries are found. At the same time, osteoblasts are at work in a neighbouring area, building bone on another surface of the islet of egg-shell and plaster mixture still present in a considerable

FIG. 13. Cavities bored in the tibia of the dog, filled in with a mixture of egg-shell and plaster. Invading vessels are rapidly destroying the mass of egg-shell and plaster. Simultaneously, abundant bone formation has started around the vessels, partly built on the filling material (Tarsoly 1963)

amount (Fig. 13). Even in case of larger defects (more than 20 mm dia) 14 days following operation, the cavities were found to be entirely occupied by newly formed spongious bone abounding in wide capillaries and having red bone marrow in its primary medullary cavities (Fig. 14, Tarsoly and Tomory 1963). The callus formed here disclosed a massive spongious structure 28 days later (Fig. 15). By the spongious callus formed under the influence of egg-shell administration, the duration of healing has been reduced by more than half compared with the controls.

According to these experimental results Tomory and Tarsoly employed the mixture of egg-shell and plaster in human material with encouraging results (see p. 251). In possession of these data, the question raised by Böhler whether there is any means to promote callus formation can be answered in the affirmative.

IMPORTANCE OF VASCULARIZATION IN OSTEOGENESIS

The effect of egg-shell described above is double: partly it has a strengthening effect on bone, and partly it stimulates the haematopoietic activity of the red bone marrow formed around the vessels. It should be borne in

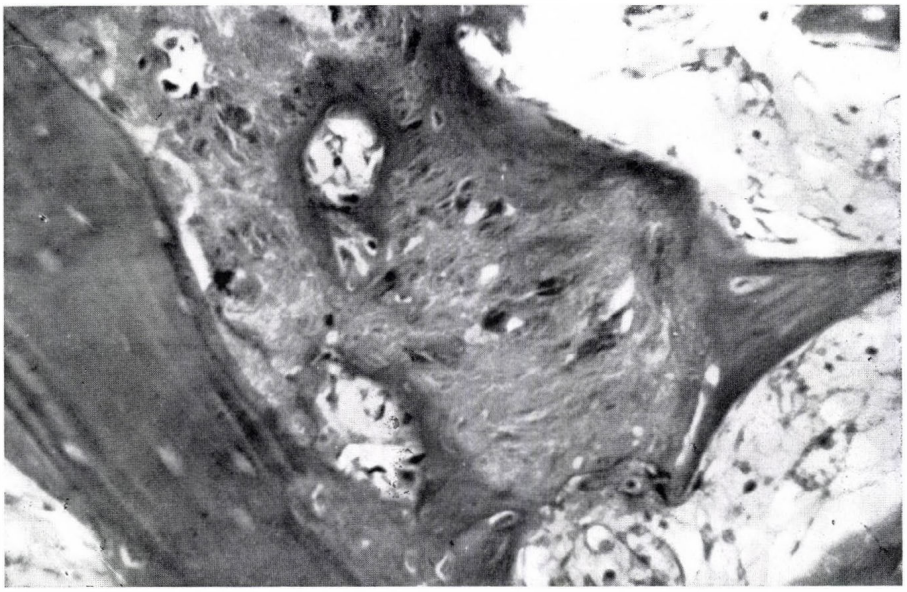

FIG. 14. Bone defect (tibia of the dog) filled in with a mixture of egg-shell and plaster after 14 days (Tarsoly and Tomory 1963), a massive spongious callus has developed.

mind that the portal system of vessels reach the bone marrow passing through the bone. As shown in Figs 14 and 15, the mass of egg-shell and plaster has been surrounded by vessels which grow into the filling material and break it down. The new spongious bone is formed likewise around vessels. It can be concluded that the vessels, creating and ensuring the conditions of oxybiosis, are the basis of bone building.

As we have seen, vascular ingrowth due to the effect of adrenal extract is a preparatory stage of bone building. Bone is formed around vessels and the metabolism of the newly formed bone is likewise ensured by vessels. The two experiments, described above, are thus, particularly appropriate to demonstrate the part played by vessels in osteogenesis, and to point out the close connection between vessels and oxybiotic metabolism, adding support at the same time to the importance of vascularization emphasized in this work and demonstrated by phylogenetic and ontogenetic data as well as by experimental evidence.

SUMMARY

Phylogenetic, ontogenetic, biological and experimental examinations reviewed in this report, unequivocally demonstrated that bone requires an oxybiotic metabolism and the presence of vessels.

At sites where vessels are absent and the conditions for oxybiotic metabolism are not ensured, a more primitive supporting tissue is formed, e.g. cartilage.

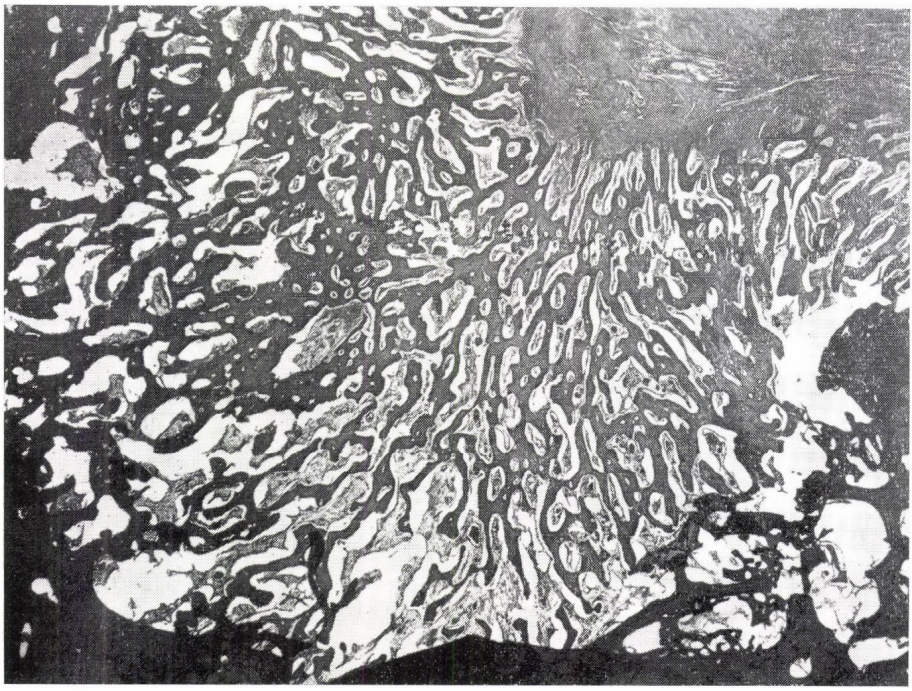

FIG. 15. Bone defect (tibia of the dog) filled in with a mixture of egg-shell and plaster: 28 days following operation, the cavity is filled up with a massive spongious callus containing red bone marrow (Tarsoly and Tomory 1963)

In the course of phylogenesis, in lower animals in which the vascular system, as condition for oxybiosis, has not yet developed, supporting tissues containing large amounts of mucopolysaccharides are found. Only after the appearance of certain endocrine glands (thyroid, suprarenal glands) and a more developed vascularization, are bone and red bone marrow, etc. found to appear.

In experiments on regeneration, in which tissular hypoxia was produced by gradual compression of the vessels of the granulation tissue, the organism was prompted to form cartilage, i.e. an avascular tissue.

Owing to the effect of complete adrenal extract, capillaries have grown into cartilage by which the conditions for oxybiosis were ensured. Consequently, the organism was obliged to form bone at such sites where no bone formation occurred up to that time, as has been demonstrated not only microscopically, but on gross anatomic specimens as well (e.g. the bony unification of the intervertebral disk with the neighbouring vertebrae).

It is known that form and function constitute an inseparable unity in the living organism. The change of form involves the change of function, e.g. if vascularization is stopped, oxybiotic metabolism concomitant with vascularization—as a function—is replaced by a lower type of metabolism which, by affecting form, brings about cartilage, a non-vascular tissue with a

mucopolysaccharide type of metabolism. According to the same principle, cartilage has been replaced by bone owing to ingrowth of vessels.

In richly vascularized tissues (state of euoxia), glucose and glycogen can be broken down through the tricarboxylic acid (Szentgyörgyi—Krebs) cycle to CO_2 and H_2O, by high energy production.

In poorly vascularized tissues (state of hypoxia), glucose and glycogen are broken down by fermentation (glycolysis) following the Embden—Meyerhof pathway, producing considerably less energy.

In non-vascularized tissues (state of anoxia), oxybiotic metabolism is reduced to a minimum, and fermentation diminishes. At the same time, large amounts of mucopolysaccharides are formed. The elucidation of the conditions of energy production in this type of metabolism will be the subject of future researches.

The conception of the present work has been formed on the basis of researches carried out for several years in the Institute of Anatomy, Histology and Embryology of the Medical University of Debrecen.

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Symp. Biol. Hung., 7, pp. 301—303 (1967)

CONTRIBUTION TO THE ANGIOARCHITECTURE OF THE CALLUS

by

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THE DEVELOPMENT of the vascular supply of the callus has been investigated for years in our Clinic, in collaboration with the Institute of Anatomy. The purpose of our investigations was to study the vascularization of calluses obtained after various treatments of fractures.

The investigations on the vascular supply of the callus were carried out after simple reposition and bloodless fixation of the fractured ends (Series I), after percutaneous diafixation (Series II) and after intramedullary fixation by nailing (Series III). Studies on the angioarchitecture of the callus after compression treatment are in progress. The experiments were conducted on 16 young dogs of approximately equal size, weighing about 7 to 8 kg. The distal third of the ulnar diaphysis was fractured obliquely by a pair of Liston's bone scissors under sterile conditions.

I. The dogs were sacrificed at different intervals following simple reduction of the fracture ends. In this way, the healing of the fracture and the revascularization occurring during the process of callus formation could be followed. After supravital injection of diluted India ink in the heart of the dog, the bone of the dead animal, denuded of its soft parts, was made transparent by Spalteholz' method and examined under magnifying glass and microscope.

After simple reduction of the fracture, the formation of new vessel buds was found to begin in the first days after the fracture. Fourteen days after the fracture, a marked capillary network was seen which displayed a fine brush-like distribution and penetrated into the site of the fracture from both ends. At this time, however, the vessels were demonstrable only in the medullary cavity the cortical part being completely avascular.

Three weeks after the fracture, the vascular system of the medullary cavity showed a much *denser pattern* than that of the cortical part. The cortical vascular system was still *poorly developed.*

Four weeks after the fracture, the connection between the vessels of the rich medullary network was already established at the fracture line, but a *marked avascular zone was still visible* in the cortical layer.

By the fifth or sixth week, the vascular system of the cortical part of the bone began likewise to develop rapidly. By this time the connective tissue bridging the gap between the two fracture ends (endosteal callus) can well be observed.

This series of experiments show that if the reduction of the fracture ends was performed avoiding the use of any foreign bodies, the endosteal vascularization preceded the periosteal one.

II. In the second series of experiments the vascular supply of the callus developing after diafixation was examined. The experiments were carried out on 15 dogs, in which oblique ulnar fractures were performed and the fracture ends were fixed by diafixation. The wire was removed after three weeks. The cortical substance was found to have been augmented to almost twice its original amount around the site of perforation of the wire. A marked periosteal reaction was seen at the fracture line. The angioarchitecture of the callus showed an interesting change. Around the diafixing wire—though it was made of a metal not hostile to tissues—a marked proliferation of blood vessels was observed. The invasion of the vessels into the fracture line was found to occur earlier than in the controls. Vascularization of the medullary cavity was found to be more abundant than that of the cortical part which was relatively poor. Thus, the presence of the diafixing wire induced an intensive, even premature development of the vascular system.

The 4-week-old specimens displayed an undesirable superabundant vascularization along the fracture line. Thus, if the diafixing needle is kept in the bone longer than three weeks, it may cause an undesirable, superabundant proliferation of the vessels in the medullary cavity, resulting in the formation of a callus consisting of soft, loose connective tissue. According to the examinations of Pap, such exaggerated periosteal reactions can be avoided if vitallium needles are used for fixation.

These examinations were carried out in collaboration with E. Hidvégi.

III. In this series the development of vascularization after intermedullary nailing was studied. Twenty dogs were used in the experiments.

Through a skin incision of about 1 cm, at the level of the tuberositas tibiae, the periosteum was cut through on the anterior surface of the foreleg of the dog, perforating the bone by a hand drill. Through the hole, a Kirschner wire of an adequate size was introduced along the whole length of the cavity. Then the bone was partially sawn through at the border of the lower and median third and broken by hand around the Kirschner wire already introduced. One week after the fracture, the callus was examined following injection with India ink. The examination revealed the haematoma and the vessels advancing from the periosteum to the fracture line.

Two weeks following the fracture, an extensive callus was formed and the vessels invading from the periosteum were found to stop on the border of the cartilage where marked glomerule-like formations were also visible.

In the 3-week old specimens, the ingrowth of vessels from the periosteum increased. The medullary arteries appeared to run towards the fracture ends.

Four weeks later, the ingrowth of vessels, both from the medullary cavity and the periosteum, was more vigorous, even displaying anastomoses between the two vascular systems.

Six weeks after the fracture, the invasion of the endosteal and periosteal vessels exhibited a further increase. The number of anatomoses was also greater.

Eight weeks after the fracture, the vascularization between the fracture ends was similar to that of the normal, intact bone.

SUMMARY

On the basis of experimental results it can be stated that the vascularization of the callus varies according to different forms of osteosynthesis. While in Series I and II (simple reposition and diafixation) the vascularization of the callus originated from the medullary cavity, in Series III where intramedullary nailing was performed, poorly vascularized areas were observed with simultaneous formation of cartilaginous callus. In the third and fourth week a vigorous invasion of vessels occurred from the periosteum and a much slighter vascular ingrowth was observed in the medullary cavity advancing toward the fracture line. In the fifth week anastomoses were seen, and by the seventh and eighth week the vascularization of the callus became similar to that of normal bone. Thus, in case of intramedullary nailing, the ingrowth of vessels from the medullary cavity was found to be protracted, but it did not cause a marked delay in ossification.

The experiments were carried out in collaboration with I. Földes and L. Tasnády.

Symp. Biol. Hung., 7, pp. 305—313 (1967)

EXPERIMENTAL DATA ON CALLUS FORMATION

by

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It is known that numerous factors play a role in normal chondral ossification, e.g. mineral salts, (chiefly calcium and phosphorus) vitamins, hormones, mucopolysaccharides, vascularity, mechanical factors, etc. A similar situation exists also in the case of regenerative ossification, namely of callus formation. In the present work we wish to demonstrate our experimental results concerning the effect of phosphate esters and of compression. Lately, great importance has been attributed to the phosphate esters. According to *in vitro* investigations the ATP is considered as an exclusive phosphate donor in the process of calcification (Cartier and Picard 1955, Picard and Cartier 1955, Barbieri 1957a, b, Castellini et al. 1955). On the other hand, recent biochemical investigations refer to the involvement of phosphate esters in the formation of mucopolysaccharides (Leloir and Cardini 1953, Castellani and Zambotti 1956, Zambotti 1957, etc). According to Krompecher (1956, 1960) the mucopolysaccharides are indispensable metabolic products of cartilage formation, whereas their presence is particularly important in the deposition of mineral salts.

Our experiments were carried out on 132 white rats. The left femur of the rat was fractured and the broken ends were fixed by a nail. The animals were treated with 2 mg of glucose-1-phosphate, glucose-6-phosphate and ATP daily. One group of rats treated with glucose + phosphate or anorganic phosphorus served as control as well as one group of untreated animals. In each group the material was examined 1, 2, 3 and 4 weeks after the fracture.

According to our experimental results the cartilaginous callus appears even in the first week under the influence of phosphate esters. We found Hale positivity and γ-type metachromasia in the cartilage cells (Figs 1 and 2). On the other hand, in the calluses of the control animals, connective tissue could still be observed with PAS positivity. Cartilage has already formed in the callus of each group in two weeks, but after administering phosphate esters, the cartilage appears in a greater quantity, and it is in these groups that chondral ossification begins. After treating with phosphate esters, these bone bars become stronger, but the enlargement of the remaining cartilage islets is also greater (Figs 3 and 4). According to our experimental results the effect of the phosphate esters on callus formation is two-fold:

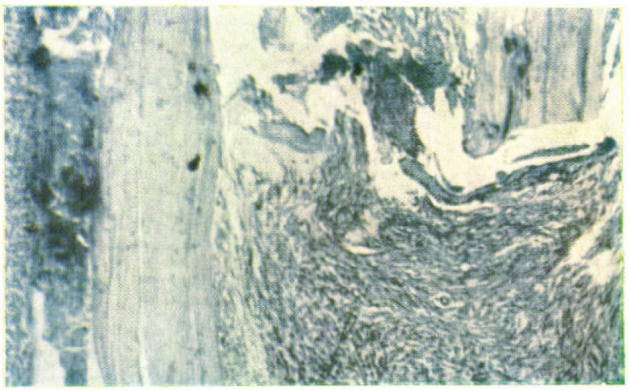

FIG. 1. One-week stage. Control group. Connective tissue callus between the fracture ends displaying orthochromasia with toluidine blue

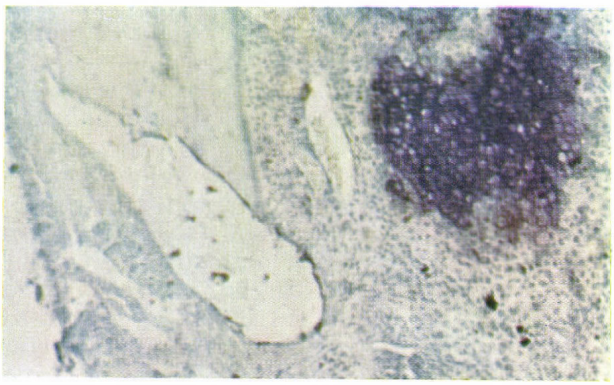

FIG. 2. One-week stage. Treatment with G-6-P. Appearance of cartilage islet giving metachromasia with toluidine blue

1. It stimulates the process of regeneration by accelerating the formation of the cartilaginous and osseous callus;
2. It prolongs the process of cartilage formation, so that the final healing is somewhat retarded.

We attempted to obtain quantitative data also on the effect of the phosphate esters. We examined the fractured bones concerning tenacity and elasticity (elasticity module) by an apparatus devised by Tarján et al. The measurements were performed according to the method of Tarján et al.

As a result of the data obtained, the 'self-control' value was calculated, i.e. the relative value of the fractured and intact leg of the same animal and the 'comparative control' value, representing the relative values obtained in the fractured legs of the rats of each group.

According to our results the treatment with various phosphate esters increases the tenacity of the intact and fractured bone, though not significantly, as compared with that of the controls and those treated with G + P (Table I).

FIG. 3. Four-week stage. Control. New spongious bone between the fracture ends. Stain: haematoxylin-chromotrope

FIG. 4. Four-week stage. Treatment with G-1-P. The new bone trabeculae are denser. Stain: haematoxylin-chromotrope

TABLE I

Values of tenacity of callus

Group	Average value of fractured leg (kg/mm^2)	Average value of intact leg (kg/mm^2)	Self-control value	Comparative control value
G-1-P	3·13	4·24	0·73	1·22
G-6-P	3·15	3·10	1·01	1·23
ATP	3·25	4·45	0·73	1·27
G + P	2·67	3·22	0·82	1·04
Control	2·55	3·44	0·74	—

This increase was not observed in the elasticity modulus (Table II).

TABLE II

Values of the elasticity modulus of callus

Group	Average value of fractured leg (kg/mm^3)	Average value of intact leg (kg/mm^3)	Self-control value	Comparative control value
G-1-P	2·251	3·709	0·62	0·87
G-6-P	2·446	3·377	0·72	0·94
ATP	2·650	3·756	0·71	1·02
G + P	2·952	3·506	0·84	1·14
Control	2·575	3·666	0·70	—

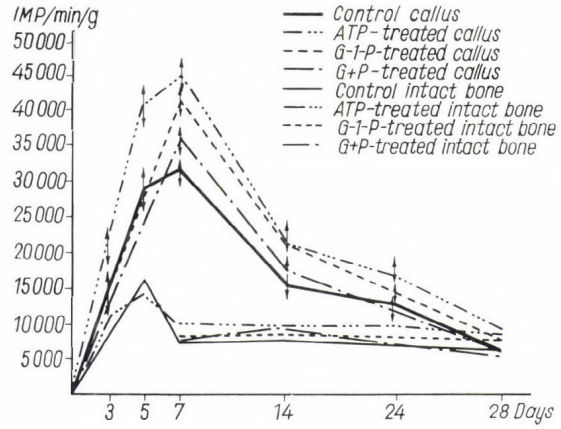

FIG. 5. Uptake of radioactive sulphate in the callus

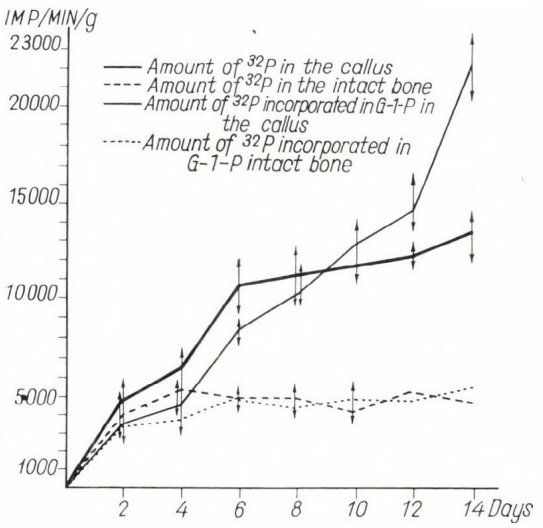

FIG. 6. Uptake of ^{32}P and that of ^{32}P incorporated in G-1-P in the callus and intact bone tissue

Different isotopes were used for a better recognition of the mechanism of effect of the phosphate esters. On the artificial callus of rats we examined:

1. uptake of radioactive sulphate;
2. uptake of ^{32}P incorporated in G-1-P compared with the control ^{32}P;
3. uptake of ^{32}P after pretreatment with ATP.

The treatment of the phosphate esters increases the uptake of ^{35}S by 30 to 40% in the callus tissue. The maximum is found approximately after one week. The arrow indicates the scattering. The increased uptake of radioactive sulphate refers to the formation of acid mucopolysaccharides (Fig. 5).

At the beginning there is no difference in the uptake between ^{32}P incorporated in G-1-P and control ^{32}P; later on the uptake of ^{32}P incorporated in G-1-P begins to increase from the tenth day (Fig. 6). In the third series, in the first week, no difference was found in the ^{32}P incorporation of callus between the control rats and those treated with ATP. In the fourth week the ^{32}P uptake of callus ofrats treated with ATP increases significantly (Fig. 7).

According to these experiments with isotopes the results with ^{35}S gave contrasting curves (Fig. 8). At the beginning the sulphate uptake increases, owing to the effect of the phosphate esters. As time elapses—parallel with the replacement of cartilaginous callus by bony callus—the activity

FIG. 7. Uptake of ^{32}P in callus and intact bone tissue in controls and after pretreatment with ATP

of ^{35}S decreases. The contrary occurs in the case of ^{32}P.

These data seem to indicate a two-fold effect of phosphate esters. In the initial stage they have an enhancing effect on cartilage and matrix formation, mainly by promoting the synthesis of mucopolysaccharides. In later stages they enhance calcification by stimulating the effect of P-donor or the development of template. Finally, the effect of compression of the mechanical factors will be discussed. The different investigations and results raised a few problems: the methodological question of the creation of a fix, permanent pressure and the necessity of more extensive histological and histochemical investigations. The work of Schenk and Willenegger (1963) concerning the healing of the compact bone fracture proved to be inspirational. Relying on the strength of these findings we determined to carry out experiments concerning the effect of pressure upon the healing of fractures. Experiments on fix pressure were performed on dogs as follows:

1. The transverse wire was applied as near as possible to the fractured ends to enable us to bring about pure pressure force;

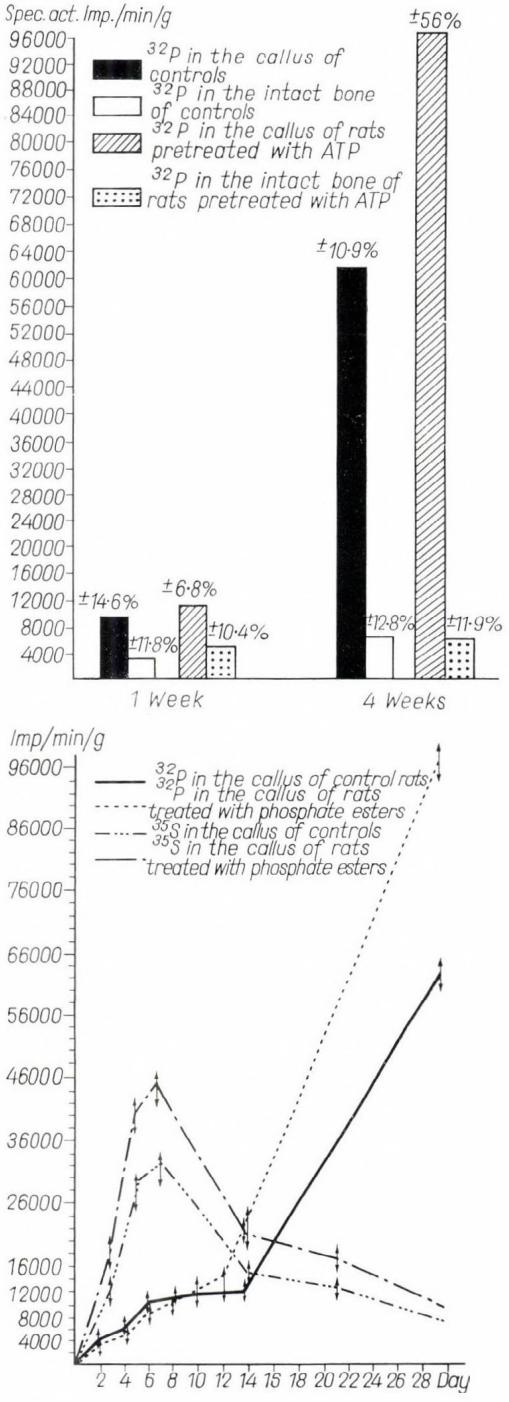

FIG. 8. Graph showing the experimental results obtained with radioactive sulphate and phosphate

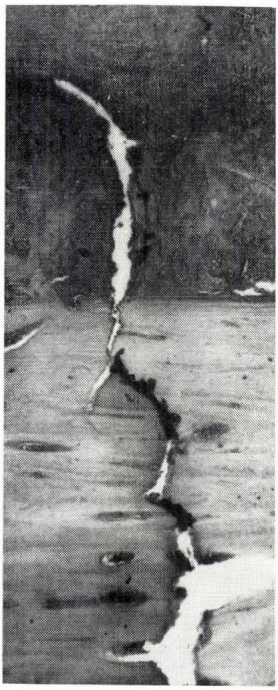

FIG. 9. Compression treatment. Ten-day stage

FIG. 10. Fourteen-day stage

2. Two splints were employed which decreased the possibility of the dislocation of the fracture ends;
3. A V-shaped fracture was made which is also favourable for the precise setting of the fracture ends.

The apparatus was applied directly after the operation. With the help of the bilateral spring, a pressure of 8 kg was exerted on the fracture ends. The animals lived for 10, 14, 21, 28, 35 and 42 days after the operation.

RESULTS

Stage 10 to 14 days: the initial period of the formation of the callus The compact bone fracture-ends conform entirely, so that only a minimal haematoma gets between them (Fig. 9). The compression is the greatest here, because the wire leans on the hole and the entire weight is brought to bear in the compact bone. Within the formation of the spongious bone gets under way (the occluding callus), and the anchoring callus consisting of the spongious bone also appears periostally. Between the spongious bones connective tissue is found, and on the 15th day, chiefly externally, smaller cartilaginous islets, too (Fig. 10).

FIG. 11. Twenty-one-day stage

Stage 21 to 28 days: in the extremely narrow space of the compact bone a very small amount of granulation tissue can be observed (Fig. 11), while in the 28-day-old calluses new, spongious bone can also be seen (Fig. 12). Between the internal occluding callus and the external anchoring callus a considerable amount of cartilaginous callus was formed within the colliding areas. At the internal callus the compressing force consists of two components:

1. The pressure of the spongious bones which grow towards each other;
2. The part of the pressure exerted by us affecting the spongious area.

Here the cartilage consists of fresh, young cartilage cells, shown by the vivid positivity of alcian blue and alcian green, and the γ-metachromasia with thionine.

Stage 35 to 42 days: the restitution of the fractures is completed. In the compacta between the fracture ends, new spongious bone has been formed (Fig. 13). Externally, the anchoring callus, forming from two directions, becomes contiguous also periostally with each other, and the cartilage islets diminish (Fig. 14). In the 42-day stage only a few alcian blue positive cells indicate the presence of cartilage. Internally, in a similar way, the ossification progresses gradually, the vessels invade the cartilage and the cartilage becomes substituted by bone. As a result of our findings, the following ideas are suggested:

1. Particularly when investigating the mechanical factors, it seems necessary to carry out a differential investigation of the external, the compact and the internal callus;
2. The compressing force does not produce an equal effect on the different calluses during their healing;
3. The intensity of the compressing force essentially influences the quality of the callus as described by Friedenberg and French (1952). We are of the opinion, however, that the weight values ought to be revised, because the 8 kg weight, considered to be optimal by these authors, appears to be too much in the case of the compact bone. Naturally, not only the absolute weight value should be considered, but also the size of the bone;

FIG. 12. Twenty-eight-day stage

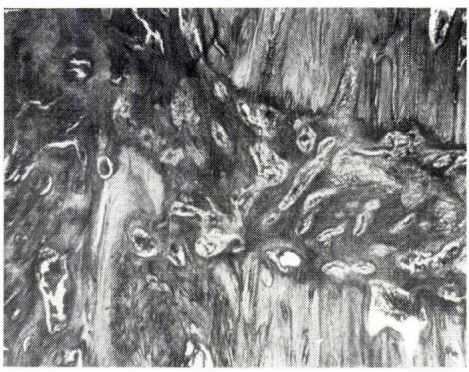

FIG. 13. Thirty-five-day stage

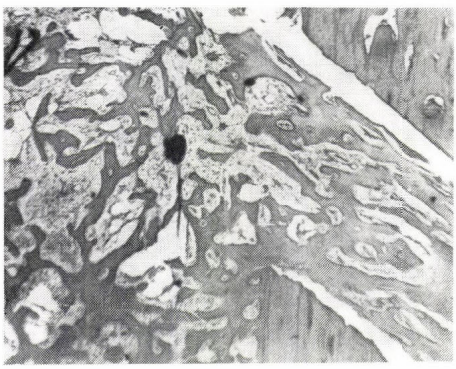

FIG. 14. Forty-two-day stage

4. A fundamental condition for the exertion of the cartilage inductive effect is the presence of granulation tissue in the initial stage of development.

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Symp. Biol. Hung., 7, pp. 315—322 (1967)

EFFECT OF ENDOCRINE FACTORS ON CALLUS DEVELOPMENT IN EXPERIMENTAL FRACTURES

by

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The role of endocrines on regeneration of bone as biological agents exerting favourable or unfavourable action has attracted increasing attention in recent research on the physiology of calcified tissues. Convincing proof has been presented of osseous anabolic effect of the hormones known to have bony targets (Asling and Evans 1956, Koskinen 1959, 1962, 1963, 1965a, b, c, Nichols 1965, Nordin 1965, Rokkanen and Slätis 1964, Uehlinger 1965, Urist and McLean 1963, Wray 1965).

This paper is based on the results of studies concerning the effects of various hormones and of their deficiency in experimental tibia fracture. The fundamental approach was that of combined quantitative study comprising tissue analysis of the callus, autoradiography with phosphorus^{32} Planimetric measurement of the roentgenological callus and examination of the clinical consolidation of the fracture. The methods and their theoretical aspects have been presented in detail in previous papers (Koskinen 1959, 1962, 1963, 1965a, b, c, Rokkanen and Slätis 1964, Uotila 1940, Uotila and Kannas 1952) to which reference is made.

In brief outline, the procedure in the quantitative tissue analysis method, also known as line sampling, is that a line is drawn in the histological section across the bone ends at right angles to the shaft and the percentage distribution of the different tissue components in the callus observed along this line is plotted to produce a diagram illustrating conditions at different stages of healing.

ACTION OF GROWTH HORMONE AND CORTISONE

Growth hormone and, in particular, growth hormone combined with thyrotropin has an accelerating effect on the healing of fractures of the long bones when injected at the beginning of bone repair. Cortisone, on the other hand, impedes the normal development of callus and osteogenesis.

The line sampling procedure revealed, compared with the controls, more vigorous formation of new bone and maturation of the callus in the test group treated with growth hormone, and even more so in the group with combined growth hormone and thyrotropin treatment. Repair started at an earlier time and progressed rapidly. At the same time, profuse vascular-

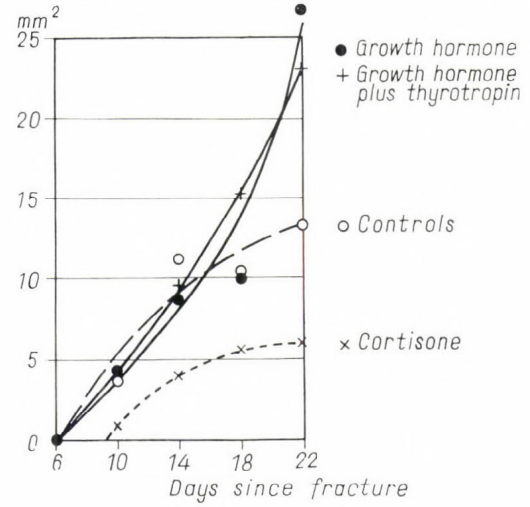

FIG. 1. Size of roentgenographic callus (planimetric measurements). Development of the callus in size in different experimental groups and in their controls during progress of bone repair; × 5. Average of 5 animals (young, growing rats)

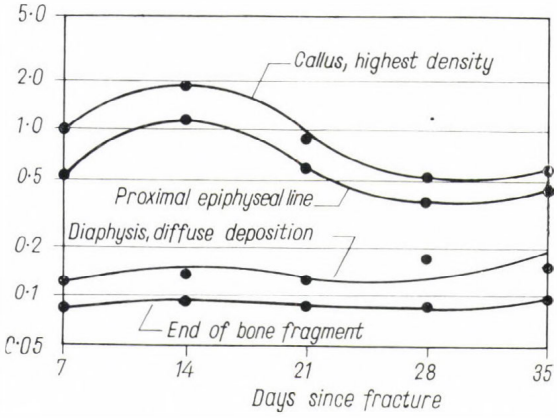

FIG. 2. ^{32}P deposition by densitometry of autoradiographs (controls). Relative intensity of radiophosphorus deposition in the callus and at specified points of the fractured bone during progress of normal bone repair

ation occurred and mature hyaline cartilage; the latter presents a fairly specific reaction in the form of peculiarly activated cells seen in the histological section (Koskinen 1959, 1965a). On the other hand, fibrous tissue and fibrous cartilage occurred sparingly. In the controls and in the group treated with cortisone, the formation of new bone was of a lesser degree and later in starting. The phenomenon was especially marked in the last mentioned group. New blood vessels were comparatively few, while immature tissue components (fibrous tissue and fibrous cartilage) were dominant.

The size of the callus seen in X-rays of the fractured tibia, as measured with the planimeter from enlarged prints (Fig. 1), increases at first fairly uniformly in the experiments with growth hormone and with the combination of this hormone and thyrotropin as well as in the control experiments.

Later, the same increase continues steadily except in the controls where it slows down appreciably. Under cortisone treatment the callus remains very small throughout the repair process.

Radioactive chemicals can be profitably used to indicate the locality and intensity of the distribution of mineral components during the course of the reconstruction process. The deposition is most prominent close to the fragments and on the epiphyseal line. Both hormonal treatment groups, namely, with growth hormone alone as well as combined with thyreotropin, displayed increased activity already at an early stage and the reaction was very evident at the final stage in the cases with combined treatment. The deposition of ^{32}P was remarkably poor in the cortisone experiments.

The localization of the phosphorus isotope coincides with the sites of new bone formation, and the strength of activity correlates with the increased vascularization of the callus. The very close topographical relation of new bone formation and vascularized areas would thus suggest that the radiophosphorus deposition is consistent with the degree of local hyperaemia.

ACTION OF SEX HORMONES AND THYROXINE

Compared with the action of growth hormone which results in an increased rate of the normal reactions constituting the repair process, androgen and thyroxine elicit effects of a similar character, though to a lesser degree and without pronounced cell activation in the hyaline cartilage. The mechanism of action of oestrogen seems to be different, owing to its calcifying effect on the callus which is evidently due to reduced resorption of new-laid ossifying tissue.

The local variations in radiophosphorus deposition were studied in some detail in the experiments concerning the hormones mentioned above, and states of hormonal deficiency, applying a method involving densitometric technique. Readings were taken at four specified spots of each autoradiograph:

1. at the point of highest density in the callus area;
2. at the epiphyseal line;
3. at the diffuse deposition in the diaphysis;
4. at the end of the bone fragment.

All readings were corrected with reference to a calibration scale.

Figure 2 illustrates the distribution of radiophosphorus deposition at different points during the repair process, which reflects the mineralization occurring in the course of normal fracture healing. The relative value of the deposition of ^{32}P in the area of the callus declines, after some initial rise, to about one-half of that found at seven days. The course of this curve is rather closely duplicated by that of the graph relating to the epiphyseal line of the fractured bone which is consistently 25 to 50% lower. Almost constant values were found throughout the period of observation for the diffuse deposition in the diaphysis and for the end of the bone fragment, the latter values being about 40% lower.

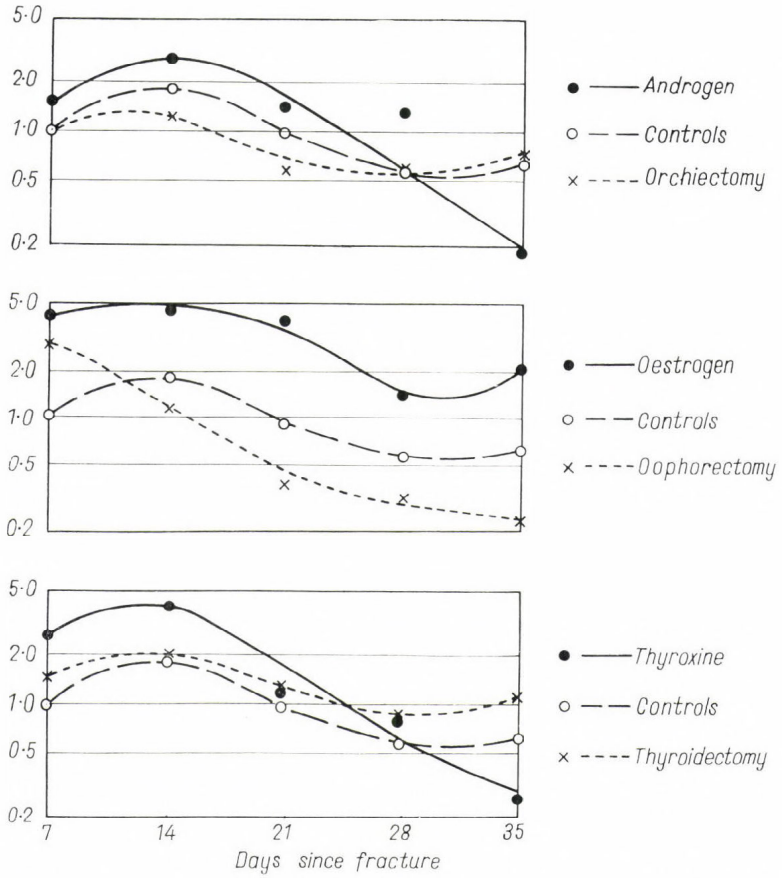

FIG. 3. ^{32}P deposition in the callus by densitometry of autoradiograph. Relative intensity of radiophosphorus deposition in the callus during progress of bone repair in 6 different experimental groups and in their controls

In Fig. 3 the densitometric evaluation of the radiophosphorus accumulation in the callus of the hormone-treated animals and animals subjected to gland removal can be seen. The corresponding curve from the control series (topmost curve in Fig. 2) has been reproduced here as dotted lines. Most striking is the effect produced by oestrogen: the radiophosphorus activity in the callus is about five times as high as in the controls. The curve has the same character as in the control experiments, running at an invariably higher level.

Androgen and thyroxine administration also seem to have stimulated the radiophosphorus deposition in the earlier stage of the repair period, though to a lesser degree than oestrogen. Towards the end of the period the values go down remarkably, even below the control value.

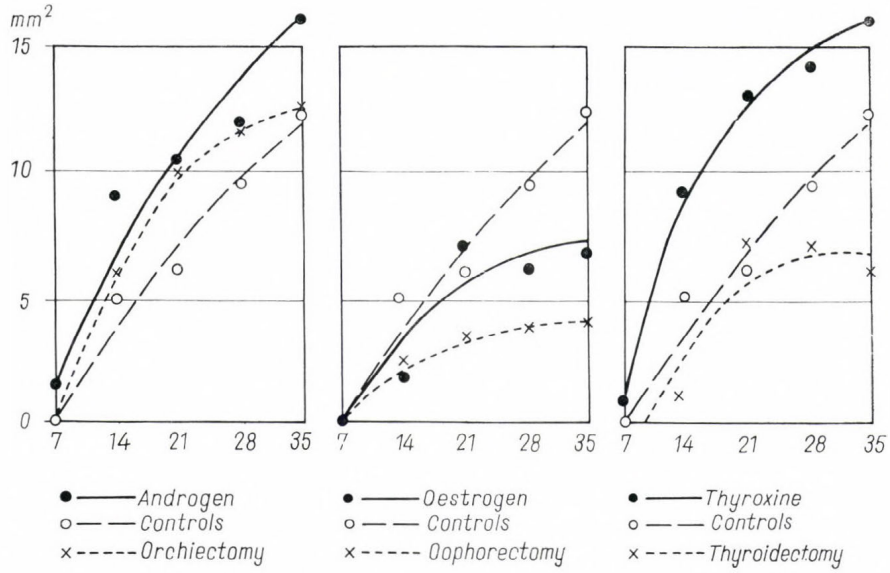

FIG. 4. Size of roentgenographic callus (planimetric measurements). Development of the callus in size in 3 different experimental groups and in their controls during progress of bone repair; × 5. Average of 5 animals (growing rats)

Among the groups involving removal of sex glands or thyroid, oophorectomy shows a distinct effect. The mineral deposition in the callus of these animals declined steadily to a final value less than half that of the controls.

Tissue-analytic approach yielded the following information. As a result of treatment with any one of the three hormones—androgen, oestrogen or thyroxine—the development of the callus reveals distinct early maturation, the formation of new bone starting one week earlier than in the controls. In the animals subjected to glandular ectomy, again immature tissue components persist during a period of remarkable length as compared with the controls. This is particularly distinct in the oophorectomy group where the callus at the final stage of the observation period still contains 26% fibrous tissue and fibrous cartilage, whereas new bone formation and vascularization have been rather scanty throughout the repair process.

Planimetric evaluation of the callus radiogram enlarged five times revealed the following trends (Fig. 4). In the androgen and thyroxine groups the size of the callus exceeded that of the controls from the very beginning of the observations and throughout the course of repair. The initial increase was more abrupt under thyroxine treatment than in the androgen-treated animals.

Essentially different from the foregoing findings is the comparatively small size of the callus in the oestrogen group which remains far below the controls even at the late stage of repair. Moreover, the sclerosis observed in the radiogram during reconstruction in the area of the fractured bone

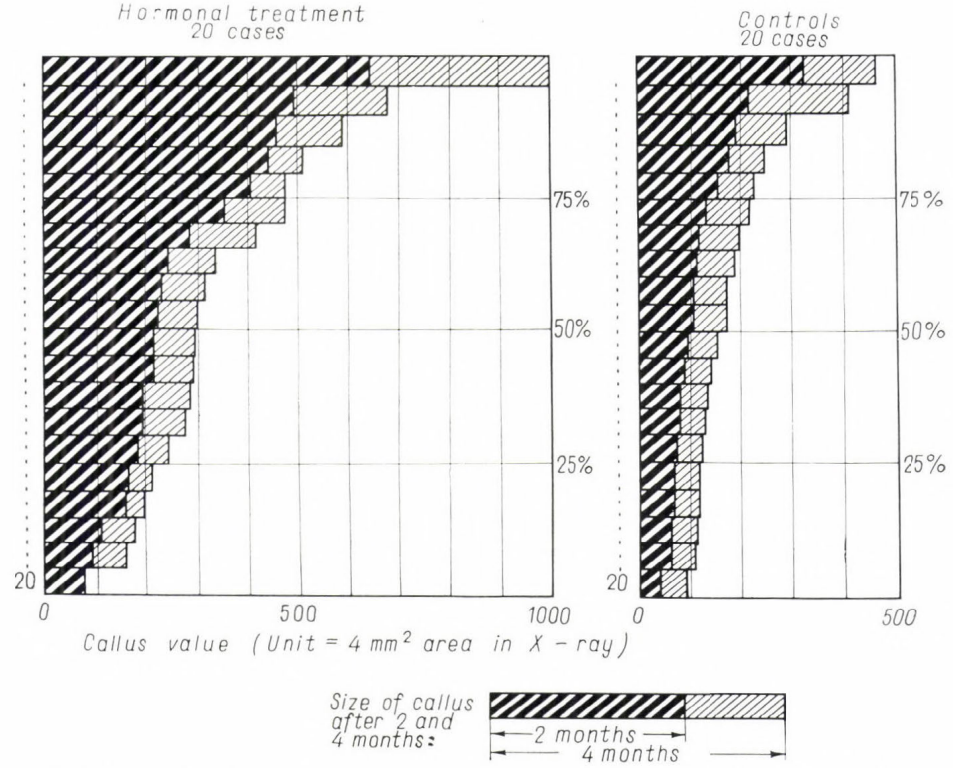

FIG. 5. Development of callus. Cumulative distribution diagram showing the size of the callus in human patients after 2 and 4 months of fracture healing in a series of 20 femur fractures with combined treatment by growth hormone and thyrotropin, and a control series of 20 similar fractures without hormonal treatment. The callus of the hormone-treated patients is larger in absolute quantity and, above all, reaches maximum size at an earlier stage than in the controls

surfaces was more clearly noticeable in the oestrogen group than in the other two hormone-treated groups.

Oophorectomy and thyroidectomy caused distinctly smaller size of the callus throughout the repair process than in the controls, while no such effect was noted upon orchiectomy. As a phenomenon common to all animals subjected to gland removal, osteoporosis was observable in the radiogram of the fractured bone.

ACTION OF HORMONES IN MAN

The influence of combined growth hormone and thyrotropin treatment has proved to be particularly reflected by the values characterizing the calcium and phosphorus metabolism in cases of human fractures or pseudanthrosis of long bones (Koskinen 1963, 1965a). Compared with the controls,

urinary calcium and phosphorus excretions decrease as does the calcium content of the serum, while serum phosphorus and alkaline phosphatase activity are slightly increased. These changes in hormone-treated cases show that accelerated mineral deposition and osteogenetic activity prevail during the metabolic stage of the repair process (Koskinen 1965a).

A highly condensed review of the principal results recorded in a series of diaphyseal fractures of the femur may serve to illustrate the point in question. The hormonal treatment consisted of 25 mg growth hormone plus 2 U.S.P. units thyrotropin given every second day during periods from 21 to 75 days. In two-thirds of the cases a fresh fracture was concerned, while the other third involved non-union or delayed union with earlier attempts to achieve bony union. The control series comprised fractures of the femur which were treated without hormones, but otherwise, according to the same surgical principles as the hormone-treated series.

Quantitative study of roentgenological callus (Fig. 5) revealed that the size of the callus in the hormone-treated series, two and four months after the onset of healing, was about double that of the controls. It is particularly to be noted that the callus of the hormone-treated patients was not only larger in absolute quantity but, above all, it was formed and reached maximum size at an earlier stage than in the controls.

The progress of healing in this material was judged by the percentage of the patients whose fracture had consolidated clinically and roentgenologically within 1 to 8 months. On this basis, the regeneration of bone in the controls was fairly constantly delayed by about two months in comparison with the hormone-treated series. Also the 10 cases in which non-union or delayed union existed when the hormonal treatment started, healed about one month sooner than the recent fractures of the cases without hormonal treatment. Equally remarkable is the fact that 15% of the controls experienced no healing within eight months, against 1·5% in the hormone-treated series.

SUMMARY

It is shown by various methods, including quantitative tissue-analytic study of the callus component distribution, autoradiography with ^{32}P and planimetric measurement of X-rays, that the healing of long bones can be controlled to a remarkable extent by certain hormones.

Growth hormone, particularly in combination with thyrotropin, has a powerful osteogenesis-increasing effect. A similar, though less strong effect is noted with androgen and thyroid hormone. The mechanism of action of oestrogen is different owing to its characteristic calcifying influence seen in the repair process and in the development of callus. Oophorectomy and thyroidectomy delay the maturation of callus, while the influence of orchiectomy is inconclusive in this respect.

The accelerated rate of fracture repair correlates with similar findings in cases of human patients under combined growth hormone and thyrotropin treatment, which results in increased mineral deposition as corroborated by metabolic studies, in osteogenetic activity, more rapid increase of callus size in the active stage of healing and in more rapid and dependable bone union as compared with the controls.

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Symp. Biol. Hung., 7, pp. 323—328 (1967)

USE OF HOMEOPLASTIC BONE SCREW IN THE TREATMENT OF PSEUDOARTHROSES

by

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PSEUDOARTHROSES are not inclined to spontaneous healing and their pharmacotherapy promises no improvement. Therefore, a number of operative solutions was elaborated for their treatment: the refurnishment of the pseudoarthroses, bone splintering (Kirschner 1949), turning of the bone splint (Lexer 1924), drilling up (Beck 1949), compressing of the ends of the pseudoarthroses (Greifensteiner 1947), riveting of the marrov space with the refurnishment of the pseudoarthrosis (Küntscher 1955, 1964), implantation of bone marrow (Máthé 1960), and installing autoplastic or homeoplastic bone splint (Phemister 1957). On account of its simplicity and reliability the last is one of the most wide-spread procedures.

In many cases the classic operative solution of Phemister (1957), in which the bone splint is simply placed under the periosteum, is not satisfactory, but it is necessary to fix it there, particularly if we want to avoid shortening by the implanted bone splint, or if the stronger bone splint is exposed to mechanical wear. Most frequently screws are used for the fixation of the bone splint.

Metals implanted in the body are in many cases not indifferent, particularly in places where ossification is undeveloped (Berentey 1956, Böhler 1950, 1955, Glauber 1958, Littmann 1956). Therefore, many attempts have been made to apply bone as a fixing material in the form of bone rivets or bone screws. Lexer (1924) and Albee carried out bone screwing. Kasth in 1938, Bürkle de la Camp in 1953, Braitner in 1955, and Schwier in 1957 and 1960 reported ostheosyntheses made with bone screws. The bone set in for fixation secures internal fixation besides the plaster of Paris bandage. Even the macerated bone, prepared in several ways, contains the factors necessary for osteogenesis, and it can be sterilized and stored safely. There is no need to remove the fixing material. On account of their properties mentioned briefly, the bone screw and bone rivet are particularly apt for fixation and, at the same time, as biological media in cases of pseudoarthroses (Bálint 1958, Trafas et al. 1954, Glauber 1958, Krompecher 1936, Phemister 1957, Lange 1949, Zetkin 1958).

The fixation with bone screw could not come into general use, because the sterilizing and preserving procedure employed by most authors made the removal and preparation of bones possible only under sterile conditions. This circumstance made the preparation of the screws very difficult.

We remove the bone from the fresh cadaver under non-sterile conditions in the dissecting room. Our screws are prepared by a medical mechanic from tibia corticalis, then they are sterilized in 1% β-propiolacton water solution. This procedure, according to the data in the literature and our own experience, is perfectly reliable, and does not influence the biological value of the bone (Gerald et al. 1955, Brandstein and Kiszel 1960, Máthé 1960, Trafas et al. 1954, Záborszky and Nyerges 1962).

The question may arise whether the bone screw is suitable for the desired mechanical application. Also in this respect we have carried out investigations at the Technological Department of the Technical University, Budapest (Jáni). The core diameter of the smallest screw of 4·3 mm thickness used by us was 3·5 mm. Here the average value of the shearing resistance was 165 kg. The shearing resistance of the strongest vitallium screw is 7 to 8 times that of the bone screw. The snapping of the bone screw of this dimension needed 116 kg energy. The snapping resistance of one screw thread amounts to 72·5 kg.

The assimilation of the bone is the more rapid, the bigger the implanted surface is in contact with the host bone. The surface of the bone screw used by us on 1·5 cm length is 6·6 cm^2, while the surface of a cylinder of the same thickness is only 1·9 cm^2. The cutting of the screw thread increases the surface almost four times.

According to the above consideration we introduced in our Department the use of bone screw in the treatment of pseudoarthroses. We partly bridge over the pseudoarthroses directly with the bone screw, e.g. the pseudoarthrosis of the internal ankle, and partly fix with it the bone splint bridging over the pseudoarthrosis. We refurnish the pseudoarthrosis only if reposition is needed, otherwise we only bridge over the pseudoarthrosis with the inlaid bone.

The site of the bone screw is prepared with a preparatory drill of the same dimension, then we drive in the screw with a key that can be fitted on the head of the screw. After the installment of the screw, we cut down the superficial part of the screw head with pliers and polish the surface smooth with a file. After the operation we fix the limb in the normal way and for the usual time with a bandage of plaster of Paris. The postoperative treatment is made in the way generally known.

The bone screws installed assimilated undisturbed in 6 to 8 months. We experienced no complication. So far we have carried out operations in cases of pseudoarthroses of the upper arm, forearm, thigh bone, shin and internal ankle in 21 cases, partly with bone screws only and partly combined with bone splints. In all cases we experienced bone healing. In addition, we made with bone screw osteosyntheses as well, with similar good results (Záborszky 1962).

CASE REPORTS

D. J. male, 22 years. In November 1960 he suffered a closed fracture of the right forearm with dislocation. Reposition, plaster of Paris fixation and physicotherapy were applied. On the ulna a pseudoarthrosis developed (Fig. 1a) which was operated in July 1961. We placed a bone splint beside

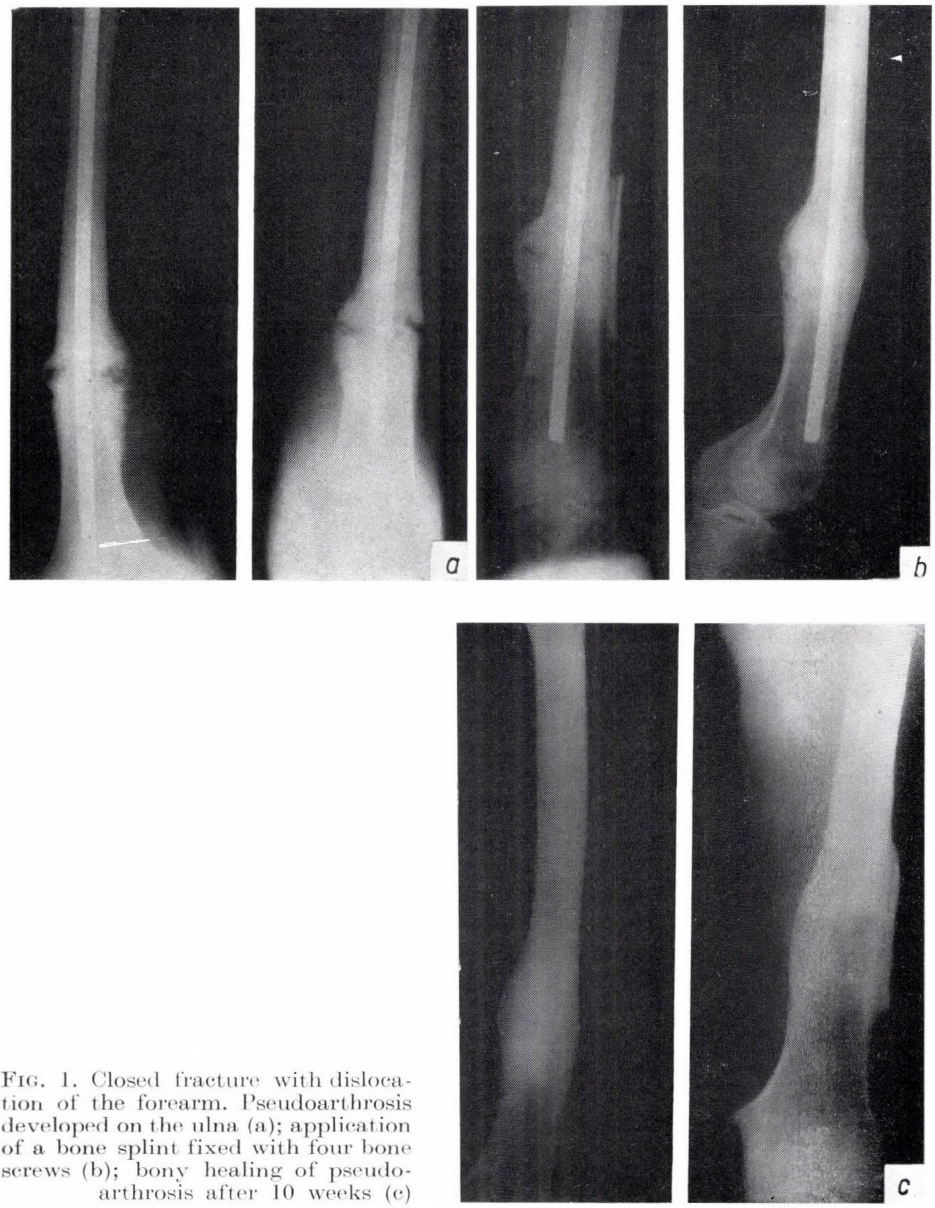

FIG. 1. Closed fracture with dislocation of the forearm. Pseudoarthrosis developed on the ulna (a); application of a bone splint fixed with four bone screws (b); bony healing of pseudoarthrosis after 10 weeks (c)

the pseudoarthrosis fixed with four bone screws (Fig. 1b). Functional postoperative treatment was given. The pseudoarthrosis showed a bony healing after ten weeks (Fig. 1c). In the seventh month the installed bone splint

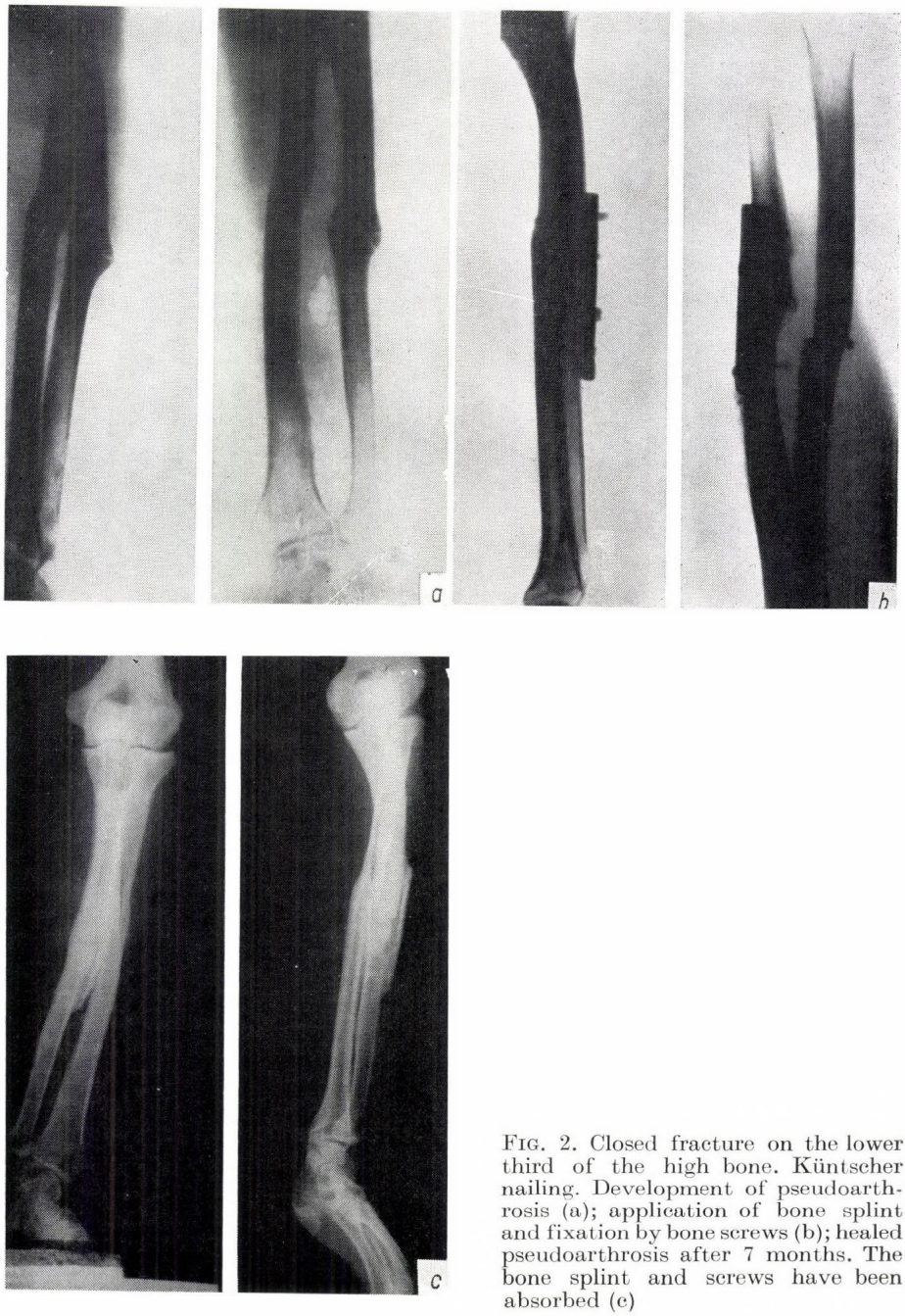

FIG. 2. Closed fracture on the lower third of the high bone. Küntscher nailing. Development of pseudoarthrosis (a); application of bone splint and fixation by bone screws (b); healed pseudoarthrosis after 7 months. The bone splint and screws have been absorbed (c)

and the bone screws became mostly incorporated. The function of the arm is perfect.

P. J. male, 28 years. He suffered a closed fracture on the lower third of the thigh bone as a result of an accident. In a country hospital riveting of the marrow cavity, after Küntscher, was carried out. A pseudoarthrosis ensued on the femur (Fig. 2a). In May 1961 we placed a 14 cm long bone splint beside the pseudoarthrosis fixed by bone screws. The rivet of the marrow cavity was left in its place (Fig. 2b). After seven months the bone splint and the bone screws assimilated, were absorbed and the pseudoarthrosis healed (Fig. 2c). After that we removed the rivet from the marrow cavity. The function of the limb is perfect.

SUMMARY

In the treatment of pseudoarthroses it is necessary to complete the usual procedure of Phemister with an internal fixation in numerous cases. Some of the fixing materials, however, have a deleterious influence on the healing of the bone. Therefore, in cases of various pseudoarthroses we have so far carried out operations in 21 cases, partly with homeoplastic bone screws alone or combined with bone splints. The bone screw secures internal fixation in addition to its osteogenetic ability. The bone screws became absorbed within eight months leaving no trace. No complication was encountered. The fixation and postoperative treatment of the limbs were carried out in the usual way.

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Symp. Biol. Hung., 7, pp. 329–334 (1967)

MECHANISM OF THE PHEMISTER OPERATION

by

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THE SCENE of evolution of the locomotor system and of all movements of the individual is the sphere of gravitation. On viewing the moving organism as a whole, the determinative effect of gravitation is concealed with automatisms obtained by the accommodation of the central nervous system. However, when the elementary phases of fracture healing are studied and the fracture is characterized by the criterion of abnormal movement, the role of gravitation once again becomes conspicuous. The dynamic balance of muscular function with gravitation resulting in posture is attained in the presence of intact skeleton and in that of neuromuscular structures functioning coordinately. In case of the lesion of any component or in quantitative changes, gravitation has a decisive role in the development of the prevailing situation. Extraordinary movement is also brought about by these changes in the broken part of the limb. 'Extraordinary movement' means an unusual stimulus through the afferent informations in the corresponding part of the extrapyramidal field. The answer—coloured also by numerous superpositions of pain reflexes—will again be a relative state of balance: the factor of spastic muscular actions and of the force of gravitation acting on the limb. Since there are no developed reflex mechanisms to stabilize such a position (named pathways in neurophysiology) even after reduction, mainly biphasic movements of small amplitude remain at the site of the fracture, called inner movements or micromovements by clinicians. This phenomenon is marked in the other type of conservative treatment of fracture, namely in the treatment by extension. Paradoxically, these two methods could be called the physiologic methods of bone healing, and both are characterized by the above-mentioned micromovements at the same time. Fractures in the world of animals, or fractures of human limbs diagnosed late or not at all—where relatively good healing tendency can be repeatedly experienced—also heal in the presence of spastic muscular fixation or 'limb defense', and proof is scarcely needed that inner movements are present regularly. The healing of these fractures is characterized by a calcifying shadow, recognizable roentgenographically and named callus. The inner movements which characterize the physiologic healing of bone are regularly connected with cellular formations of intermediary callus. Krompecher, a research worker from Debrecen (Hungary) recognized this regularity. Conditions of movement at the fracture site develop differently if

an operative method of fracture treatment, i.e. an osteosynthesis is chosen. Morphologic signs of healing are entirely different compared with the former case. The intermediary phase of chondrodesmal callus regularly following the effect of push and pull is omitted if inner movements are excluded by stable operative inner-osseal fixation. Since roentgenograms show different characteristics in clinical cases of primary osseous bone healing and of bone healing by callus, so in clinical cases of bone fractures the dominance of one or the other type is recognizable with great probability. Besides, every limb surgeon knows the extent of movement allowed after an operation on bone which will be quantitatively recognizable in the callus shadow of certain shaft fractures. The casual link between inner movement and the clinical callus on the roentgenograms appears even more convincing when the roentgenograms of such an osteosynthesis are analysed, i.e. which became movable again after a certain period of stable, motion-free fixation. This is not rare with the so-called onlay solutions, where a really stable fixation can be achieved with the help of screws. Bone graft or metallic plate can be used alike as an onlay medium. After fracture of the onlay mediums at the unconsolidated fracture site, movements will appear, and on the callus shadow-free roentgenogram of fracture—typical of primary osseous healing—the usual X-ray phenomenon of desmal callus will be seen.

The roentgenographic analysis of the fate of such a fracture, with initial osseous and later desmal healing, suitably proves the casual link between desmal bone healing and regular inner movements, and at the same time provides joint proof of the thesis of Krompecher and Karlinger. Callus shadow is never observed in the first phase of healing, because stable inner fixation produces conditions of osseous healing. After fatigue fracture (of the onlay), the mechanic milieu of the developing inner movements means an indicator and condition for desmal healing. This starting movement is the direct reason of phenomena which, as proved by Krompecher's studies, provoked a desmal type of healing. The appearance of a shadow of callus in such a case is conclusive. According to our conception, this regularity gives an explanation for the initial phenomena of physiologic healing of bone and to recognize the therapeutic effect of a good osteosynthesis procedure.

By recognizing that the reason of fibre formation between the fracture ends is movement of a determined amplitude (Krompecher's recent biochemical studies have thrown light on the reason of fibre formation), the reciprocal effect of movement,—fibre formation—becomes apparent at once. The movements are modified in quality and quantity by the fibrous tissue, soft part callus; they gradually decrease, i.e. the fixation of the fragments will be greater and more stable. Clinicians value and use this phenomenon of 'springing' as a constantly important sign in the early phase of desmal healing. In this phase in human material, an indirect impression can be obtained of the stabilizing value of osteosynthesis at the site of fracture. The ossification of the soft tissue callus takes place in this milieu of diminishing 'springing' movement. The fibrous tissue itself changes into a bony bridge, then bone structure appears between the actual ends of fragments.

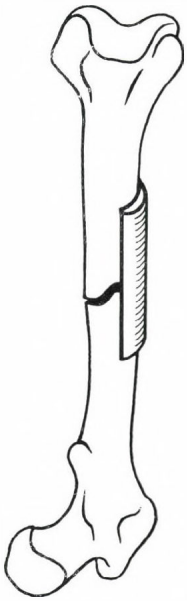

FIG. 1. Principle of the Phemister operation. On the freshened cortical surface, without exposing the tissues of pseudarthrosis, a well-adapting bone graft is applied

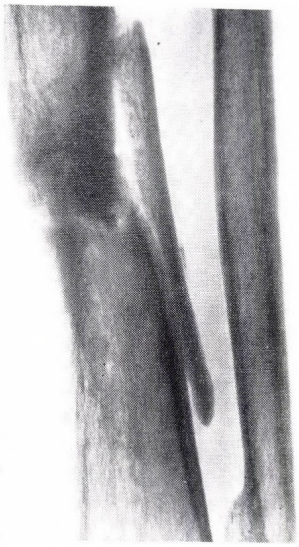

FIG. 2. Phemister operation performed in a clinical case of pseudarthrosis of the leg. X-ray picture taken 5 weeks after operation

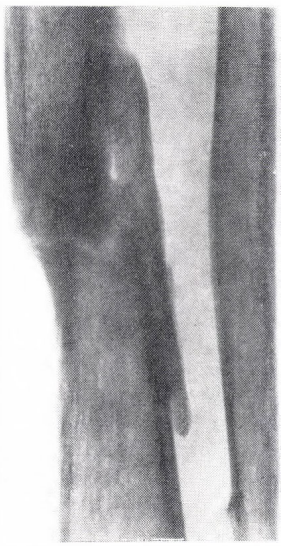

FIG. 3. Case in Fig. 2. In the gap of pseudarthrosis and at the sites where the graft was well adapted, the transformation of the callus into bone tissue is fairly visible roentgenographically

In case of an aberration in the fracture healing, as in non-union or delayed union, the characteristic 'springing' movement is never present between the bone ends in the pseudarthrosis. In a case like this the Phemister technique or the sliding inlay autogenous bone graft method is a well known procedure of healing. The essence of both procedures is to diminish the amplitude of the gross movements present with precisely fitting walking cast and to bridge the intact pseudarthrosis with a bone splint. In this respect the difference between the sliding inlay bone graft method and the Phemister procedure is only a modification without any essential difference. The sliding inlay bone graft is autogenous and not an onlay as the Phemister graft. The essence of the Phemister technique (Fig. 1) was summarized in the concise title of the paper by this prominent author: "Treatment of non-united fractures of long bones with an onlay bone graft without tie or screw fixation, or without breaking down the fibrous union." A decisive phenomenon in both procedures is the fixation of the graft to the bones of the non-union by locally formed new fibrous tissue. In early non-union or delayed union the fibrous union between bone ends formed with the help of the graft creates or at least imitates conditions of movement between desmally healing fragments (Figs 2 and 3). According

to our experience, both procedures are most reliable and strikingly rapid healing with a typically oval callus is not rare. The union in the pseudarthrosis is by scar tissue not by collagenous fibres, and the possible movements are stimulated by the newly developed collagenous fibre bridge at the ends of the applied graft. Possible movements within the plaster cast take place around the non-union as a fulcrum. Necessarily, there will be a point in the area covered by the graft where adequate movement will be present to form the collagenous fibres between the Phemister graft and the bones of the non-union. First a collagenous bridge is produced at this point which determines the conditions of movement of the non-union bone ends by the graft and which is similar in measurements to the inner movements of a desmally healing recent fracture. Although not of conclusive value, the validity of this hypothesis seems to be supported by the identity of the index of elasticity in the experimental Phemister graft and in the collagenous tissue stabilizing the experimental fracture.

In the origin and in the biology of the collagenous fibre the structure and the measurement of the surface are important factors. According to unanimous clinical experience, the desantigenized Kiel-bone graft with its great surface is most suitable for the purpose of the Phemister technique.

CONCLUSIONS

The conservative methods of fracture treatment as plaster fixation after reduction or treatment by traction and the operation method of osteosynthesis form two sharply separated, yet equally valuable procedures. The connection between the push-pull power effect of the conservative treatment and the regular appearance of the chondral-desmal transitory callus can be paralleled with the customary phenomenon of clinical roentgenology called 'calcinous callus shadow'. This clinical roentgenographic sign can be comprehended as a sign referring to histological changes in the conservatively treated fracture.

This roentgenographic phenomenon is absent, as a rule, on the roentgenograms of really stable inner fixations of fractures (osteotomies, pseudarthroses) throughout the whole process of healing. The kind of bone regeneration taking place in a totally movement-free surrounding means the development of primary osseal bone scar without the appearance of intermediary callus as proved by the scientific committee on osteosynthesis in Switzerland (Küntscher suggests the term 'direct' bone healing for this form, and that of 'indirect' form for desmal bone healing, since clinicians may misinterpret primary and secondary attributes for sterile healing or for healing under infectious circumstances). Thus, the absence of callus shadow during the whole process of healing of the fracture (osteotomy, pseudarthrosis) will prove the absolute elimination of movements and will simultaneously prove primary osseal healing. If these two rules together explain the mechanism in the healing of a fracture where after the failure of an initially movement-free fixation the roentgenographic shadow of callus formation appears, the theory can be accepted that the beginning of desmal healing is started by inner movements. The deterioration or total ceasing of the

blood supply is ultimately the direct reason of the formation of intermediary fibrous-cartilaginous type of tissue. This recognition is a recent great result of the studies of Krompecher and his followers.

On the basis of the concept described above concerning the effect of the Phemister graft and its experimental demonstration by the work of this research group and from its results, data are provided on the clinical form of desmal healing, using a biological surgical method as a model and it shows how to support callus formation 'artificially' by the kinetic conditions of desmal healing.

EXPERIMENTAL RESULTS

We wished to demonstrate, experimentally, that under physiological conditions in healing shaft osteotomy, a particle of a given standard diameter of collagenous-fibrous tissue obtainable in the third week, will exhibit almost identical physical properties, as in the case of an artificial model of a Phemister operation where the fibrous tissue of a similar surface develops between the applied graft and the bone surface of the pseudarthrosis.

On the femur of a dog, by transverse osteotomy allowing 3 cm of overriding, a soft part callus binding the fragment was produced. By resecting the whole femur in the third week with the original callus tissue intact, 3/4 sectors of the circle were resected from both ends of the femur by a motor saw. These two bone splints with slightly bowed segments were found to be bound together by fibrous callus at the 3 cm long overriding. In this callus mass a 4 mm^2 area was produced by manipulation with razor blades (see figure), and so the torsion and stretching qualities of the collagenous tissue of a standard diameter could be studied by an optimal leverage.

The experimental study of the Phemister graft took place in its fixing fibrous connection to one of the non-union fragments. A V-shaped osteotomy was performed at the middle of the femoral shaft of a dog and the distal fragment was further cut to form an obtuse V-angle. Desmal healing could be produced by an intramedullary Kirschner wire fixation for in case of a given spasm the healing of a precisely made V-osteotomy used to show rather the osseal type of direct healing. In the second week, initial springing was observed by physical examination, fibrous tissue was injured under narcosis by slight traction and moderate torsion and flexion movements of 25 to 30° in all directions. This was repeated twice every tenth day, and so the rather loose passive motility ending in about 20° jolts—typical of non-union—was produced. Then a Phemister graft, overlapping the site of the osteotomy by 4 cm on each side, having a well-fitting cancellous surface was applied, and so fibrous connection was brought about among the ends of the Phemister graft and the fixed fragments of the femoral shaft in the milieu of the system characterized by non-union motility. Three weeks after the second operation, the whole shaft-Phemister graft system was resected. After having removed the intermedullary wire, the non-union tissue was cut cross-wise with a sharp blade as far as the Phemister graft. Then the fibrous connection was sharply severed by leading the blade flatly between the Phemister graft and the contact surface of the distal fragment. By this method, an essentially

similar preparation was produced to the one used in the study of callus formation between overriding fragments. The isolation of a similar transverse sector of the callus tissue was completed—as in the case of the fracture preparation—and this was studied in the same way as earlier. The graphic representation of the regularity of the elastic demand gave a graph and its characteristics could be recognized by mathematical analysis. By a similar graph, the degree of movement following the application of the torsion force on the fibrous mass could be represented, and this again was analysed and its characteristics were revealed. A comparison of the characteristics of the two curves confirmed the identity of the material obtained either from fibrous fracture callus or from callus tissue fixing a Phemister graft.

Symp. Biol. Hung., 7, pp. 335—339 (1967)

HEALING OF PATELLAR AND TIBIAL FRACTURES

by

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Increasing attention has been directed recently to the biomechanical aspect of fracture treatment, in addition to the mechanical factors. Numerous authors (Perkins, Fairbanks, Apley, Andreesen, etc.) have given preference to functional treatment to avoid the damaging effect of the plaster bandage (atrophy, arthrofibrosis) on the soft parts and joint. The basis for this practice was provided by the observation that at certain sites of the organism (rib, clavicle, shoulder-region and knee region) the healing of fractures occurs more readily than in others. This 'segmental' property of fractures has been discussed in detail by Professor Pap (see p. 137) On the basis of this concept, a treatment employing directed active movement has been developed in our Clinic. The essence of the treatment is that at those sites of the organism where there is a tendency to fast recovery, the therapy of movement should be employed instead of immobilization. In such cases the effect of function asserts itself not only in the adequate activity of the soft parts (muscle, joint), but also an earlier healing may be obtained. Vascularization seems to have an important role in this process. A close interrelation was found to exist between vascularization and fracture healing; good healing being obtained where vascularization was likewise good, while in fractures where the vessels were damaged (e.g. in the upper third of the tibia) the formation of the callus was delayed (Figs 1 and 2).

By employing our treatment, we succeeded in restoring perfect function (capacity for work) with healing not only at the sites of the organism with good tendencies for recovery, but it was successfully applied in cases of patella fractures which have, so far, always been indications for surgery. At present, directed active movement is employed in such patellar fractures where the dislocation does not exceed 8 mm, i.e. where retinacula remained intact. Following drainage of the haematoma and application of a pressing figure-of-eight-bandage, the patient begins to move his injured leg on the third or fourth day (Fig. 3). The patients usually get up and walk in the third week. In cases of extensive dislocation and rupture of the retinacula, surgical intervention (cerclage) is performed, but no plaster cast is placed on the leg. Further on, the treatment is similar to that of non-operated patients. The average duration of healing of our 31 patients with patellar fractures was 43 days, and so the patients were able to resume work in the sixth week. With the conventional treatment employed formerly, only

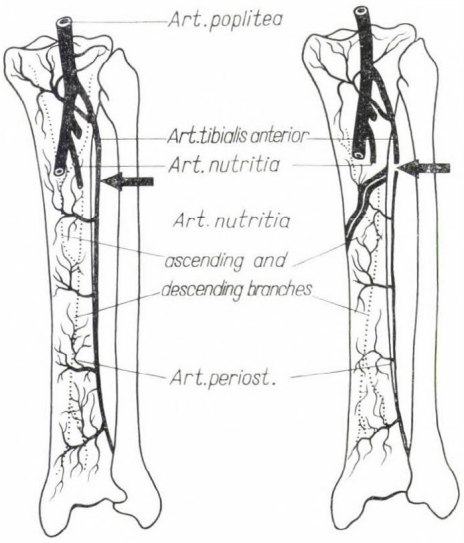


FIG. 1. Scheme of blood supply of the tibia. In case of injury of the arteria nutritia in the upper third, callus formation is delayed

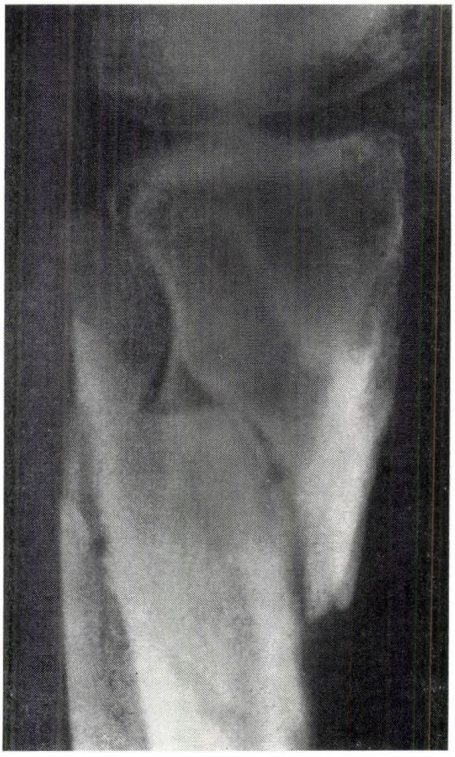


FIG. 2. Fracture of the upper third of the tibia. No callus formation is visible 10 weeks after the accident

the plaster was kept on for six weeks and the post-treatment (bath, exercise) lasted for 3 to 4 weeks.

In the experimental studies of Krompecher and Pap, patella fractures were examined in dogs where no fixation was performed. It was histologically evidenced that in such cases connective tissue developed between the fractured bone ends leading to desmal bone formation.

The concept of regarding the fractured bone as a part of its surroundings prompted us to employ directed active movement in cases of fractures of the shaft of the tibia (middle and lower third) as well. Such fractures having a reduced tendency for healing should be fixed. Here we had to solve the apparently contrasting problems that the fracture required rest, i.e. fixation, while the adjacent joint and soft parts needed moving. Rest was ensured by reposition and diafixation and by application of plaster cast on three fixed sites and extension (Figs 4 and 5). The plaster cast leaves the knee and ankle free, so that the patient can move his limb, and blood circulation and activity of the muscles and joint are not inhibited. It was surprising to see some of our patients raising their limbs on the third

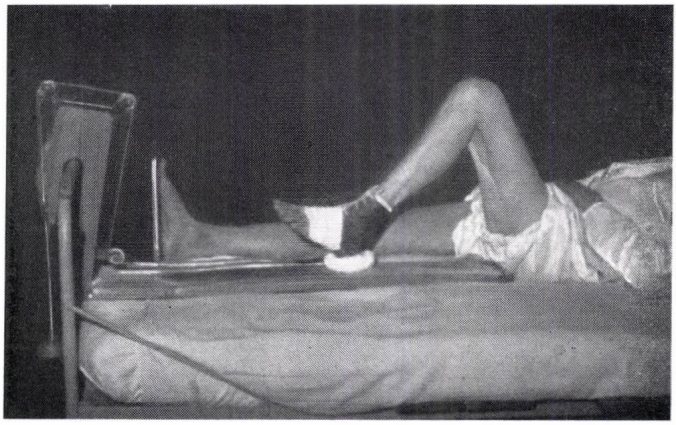


FIG. 3. Treatment of patellar fracture with directed active movement

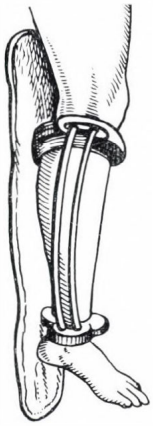


FIG. 4. Scheme of treatment of fractures of the shaft of the tibia according to Hippocrates. The principle of the treatment to leave the joint free is worthy of interest

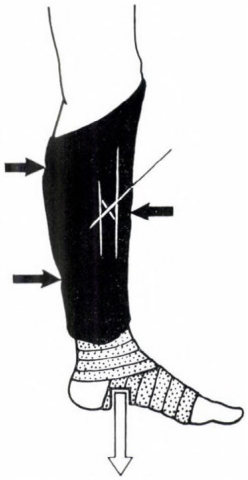


FIG. 5. Fractures of the shaft of the tibia, scheme of treatment (diafixation, extension and plaster cast). Diafixation may sometimes be discarded, since the correct adjustment of the plaster bandage with 3 points of fixation ensures adequate fixation of the replaced bone ends. A zinc-oxide bandage is applied to the leg to prevent swelling

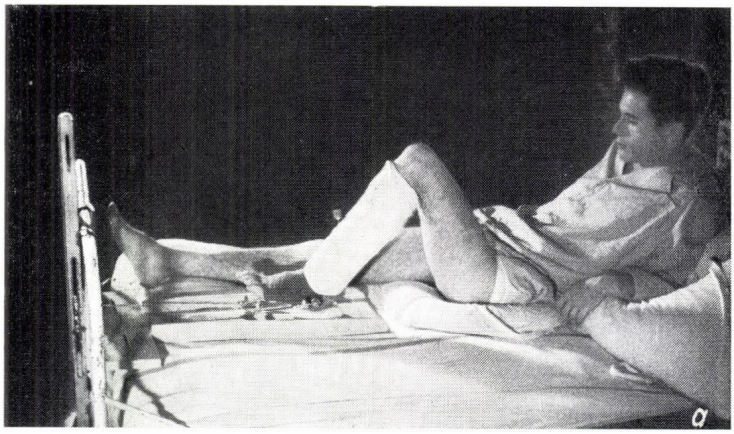


FIG. 6a.Treatment of typical fracture of the shaft of the tibia with directed active movement

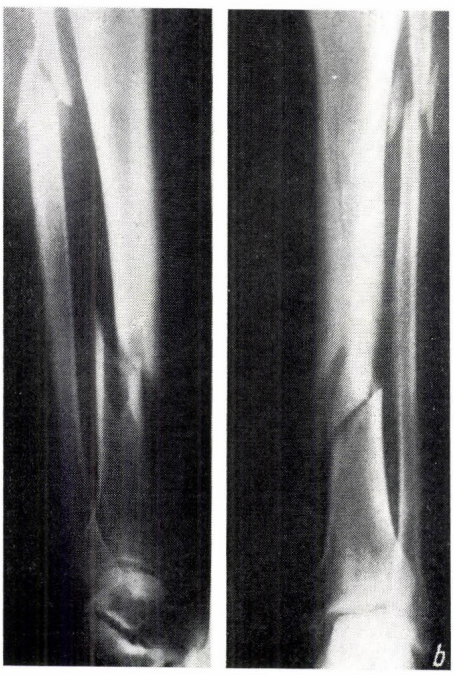


FIG. 6b. Fracture of the shaft of the tibia with typical displacement

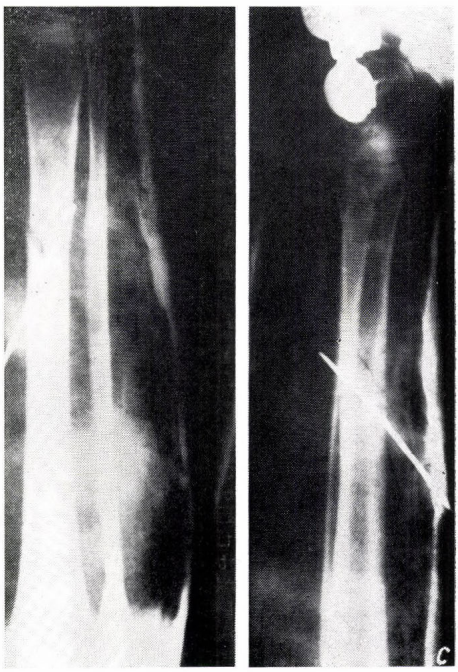


FIG. 6c. After treatment (diafixation, plaster cast and extension)

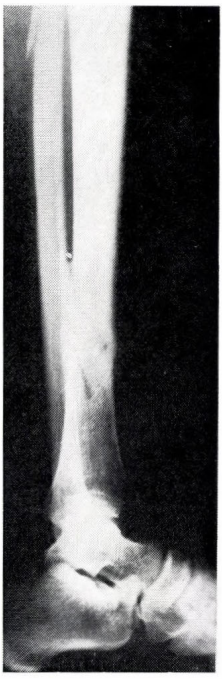
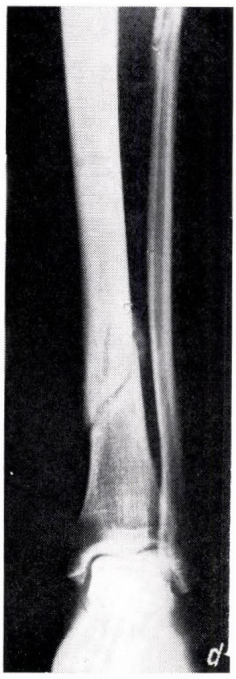


FIG. 6d. After recovery (10 weeks following the fracture)



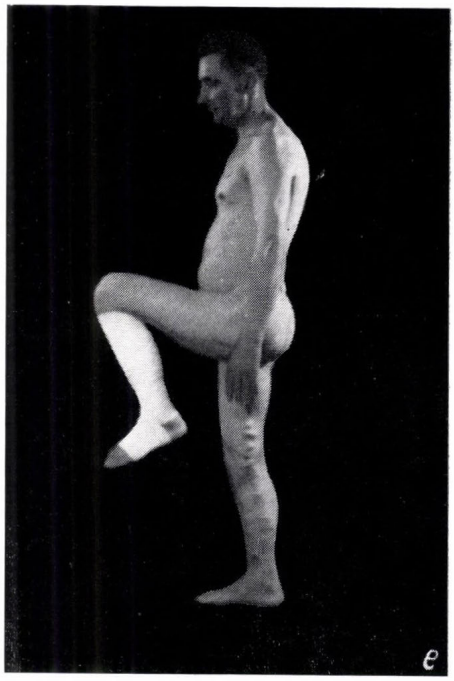


FIG. 6e. The injured leg is healed, the patient is able to work

or fourth day. This is evidence that the fractured bone ends were kept together well by diafixation. After removal of the nail, usually in the fifth week, the patients are able to walk without weight-bearing. The roentgenograms taken on the sixth week reveal good callus formation between the fractured bone ends. On removing the plaster cast in the eighth week from patients with fractures of the shaft of the tibia, they were able to resume work in the eleventh or twelfth week (Fig. 6). Thus, the question whether the duration of time necessary for healing of fractures can be shortened or not, can be positively answered. This result is due to the fact that the treatment employed by us conforms to the segmental biological milieu of the limb.

The basic principles established by Böhler are still valid in certain definite segments of the limbs.

By attaining the biological equilibrium of movement and rest, better results may be obtained in fracture healing if, in addition to mechanical factors, the biomechanical aspects are also taken into consideration.

Symp. Biol. Hung., 7, pp. 341—349 (1967)

ALLOPLASTICS AND CALLUS FORMATION

by

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THE IMPORTANCE of callus formation is evident not only in the treatment of fractures but also in orthopaedic corrections, filling up of defects and in other respects as well.

In this work we report on the effect of acrylate on callus formation. We use this material chiefly to fill up defects which have resulted from resection or excochleation of bone tumours, mostly of osteoclastomas.

The bridging of gaps is sometimes performed by employing cancellous bone grafts which are able to produce active bone besides having a stimulating effect on bone induction. However, this is not desirable in case of tumours since it increases tissue irritation present in the bone in this hyperactive state. Moreover, the grafted bone is destroyed by osteoclasts—as Janasek demonstrated at the Orthopaedic Congress in Budapest (1961). The cortical bone grafts have only an inducing effect on ossification and the lyophilized bone, though having a more reduced effect on bone induction, is rapidly broken down. In addition, the possibility of malignant degeneration is the same as in the case of cancellous bone grafts.

The unsatisfactory results experienced in filling bone defects prompted us to introduce the use of acrylate prosthesis by which the tumorous cells (viruses?) do not penetrate and it causes no tissue irritation of malignant degeneration.

In the beginning we employed acrylate in joint replacement but it proved to have a strong calcifying effect which greatly reduced the result obtained in stability. Therefore, the acrylate has been used chiefly in the diaphyseal and metaphyseal parts of long bones where, after a relatively short time, the acrylate was found to be surrounded in a sheath-like way by a periosteal callus originating from the bone. Later on this periosteal callus developed to a reaction-free complete bone tissue.

These observations have been verified histologically in 2 cases. In the first case an excision was made when the Küntscher nail used for internal fixation was removed. In the second case the material originating from beside the acrylate prosthesis was obtained by the post-mortem examination of the patient who committed suicide eight years after the operation. These cases are illustrated in Figs 1 to 15.

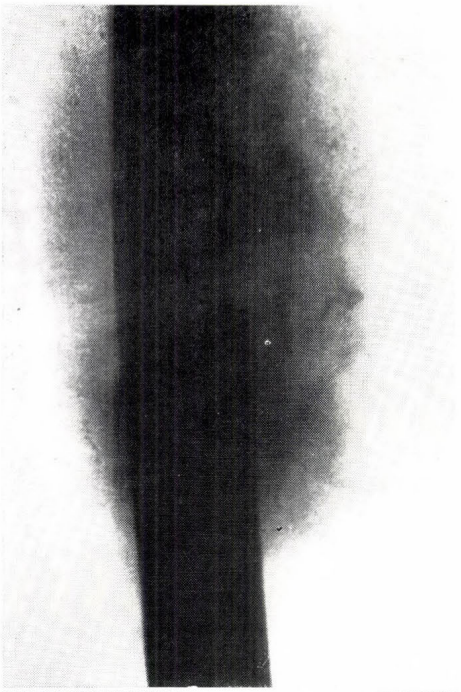


FIG. 1. Osteoclastoma of the right femur

FIG. 2. On the roentgenogram taken immediately after the operation, the Küntscher nail (a) and the external splint with screws (b) are fairly well visible (the acrylate does not give an X-ray shadow)

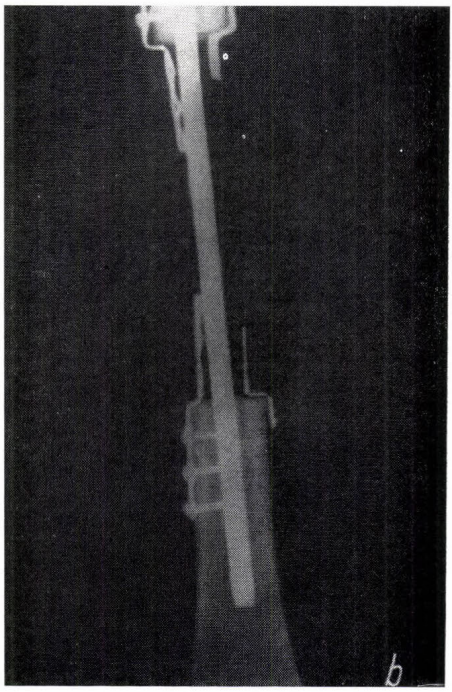
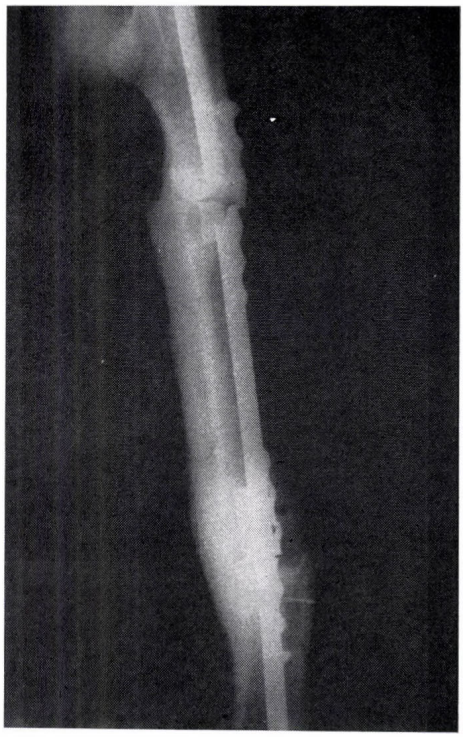


FIG. 3. After 3 years a strong and stable callus has developed adjacent to the acrylate (the Küntscher nail in the upper part of the prosthesis has suffered fatigue fracture)



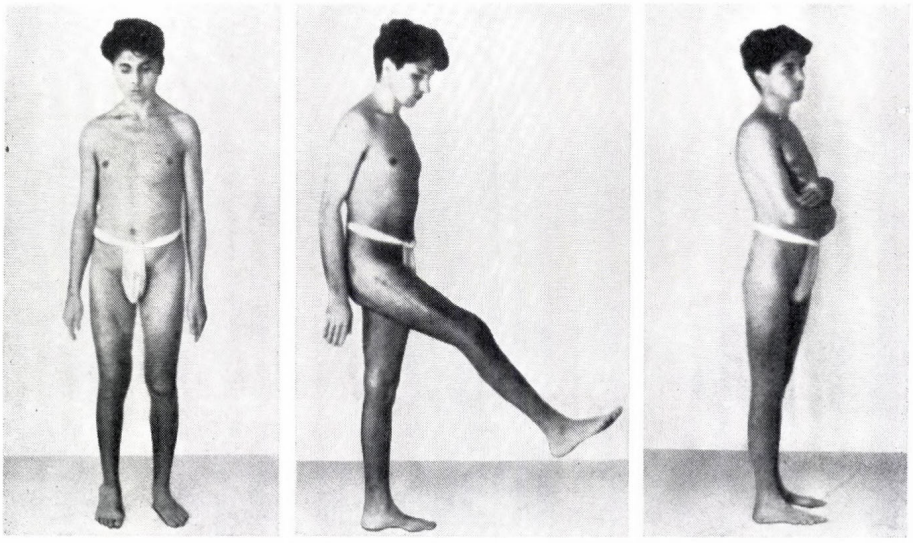


FIG. 4. Complete function of the extremity after operation

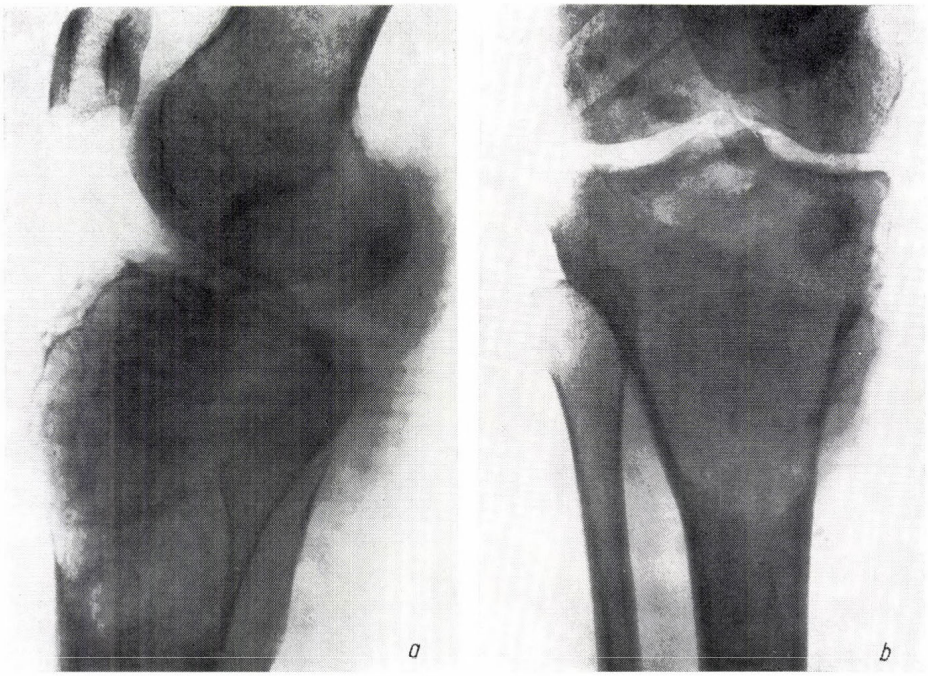


FIG. 5. Osteoclastoma almost completely destroying the condyle of the left tibia up to the cartilage border

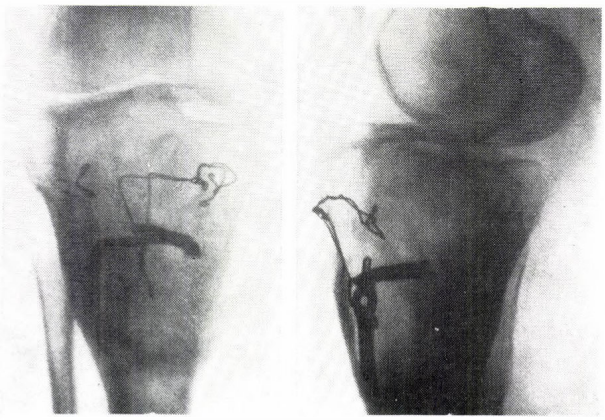


FIG. 6. One year after the introduction of acrylate prosthesis. The narrow sheath-like callus is fairly well visible

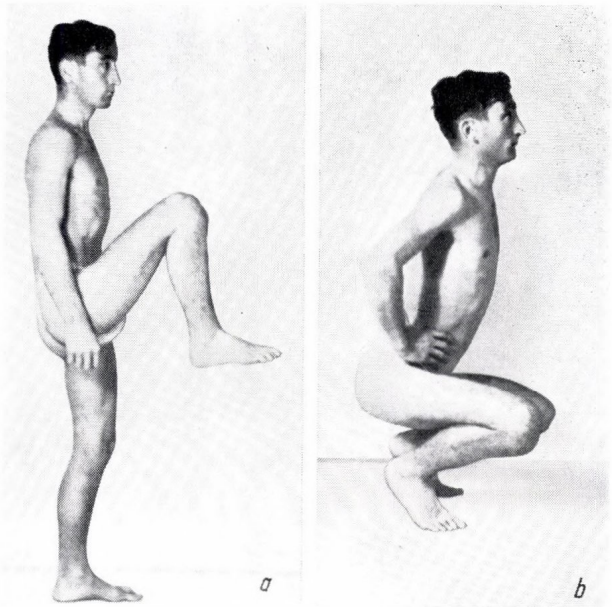


FIG. 7 Perfect knee function (the patient is employed in heavy agricultural work)

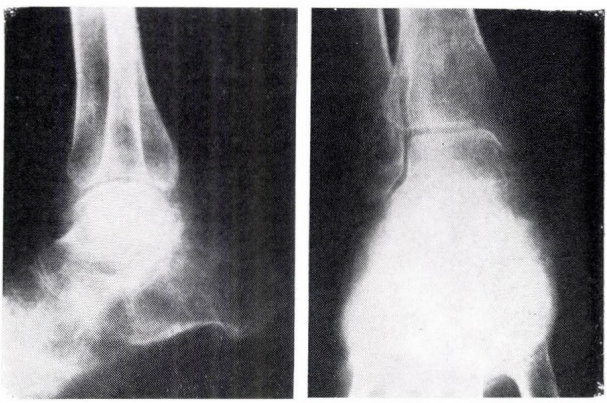


FIG. 8. Osteoclastoma of the left medial ankle destroying the bone up to the cartilage border

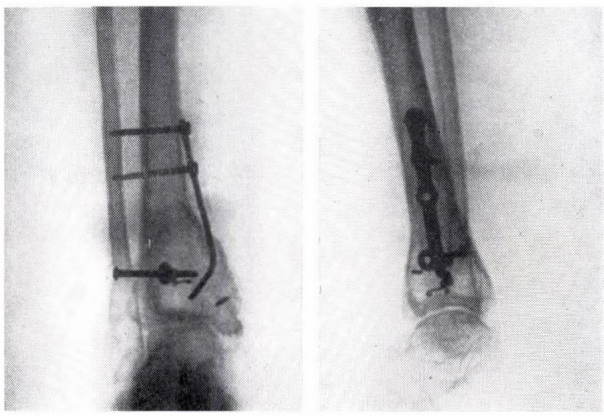


FIG. 9. Implanted acrylate prosthesis with splint and screw fixation

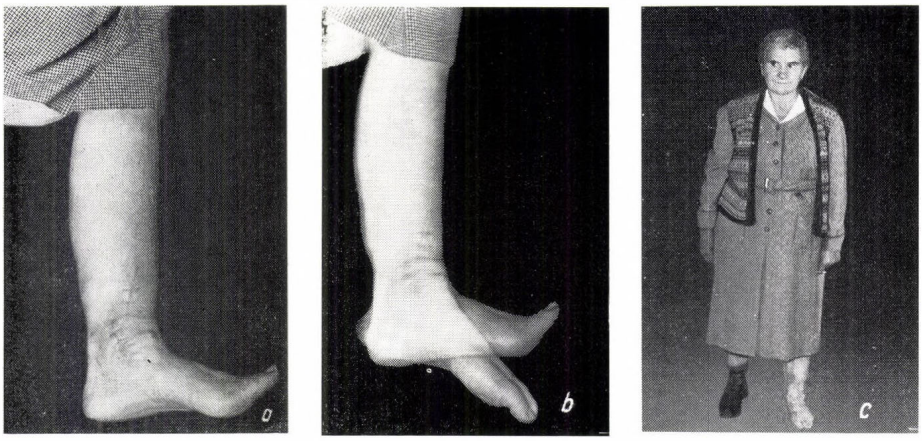


FIG. 10. Weight bearing; satisfactory function of the ankle

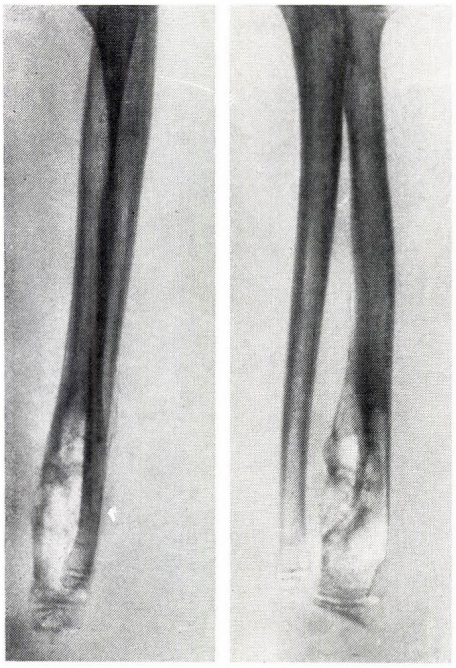


FIG. 11. Osteoclastoma of the radius in the vicinity of the joint

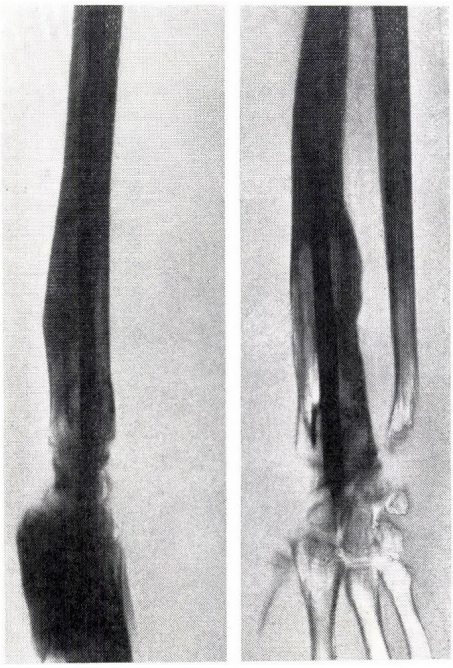


FIG. 12. Six months after operation, satisfactory callus has developed beside the acrylate

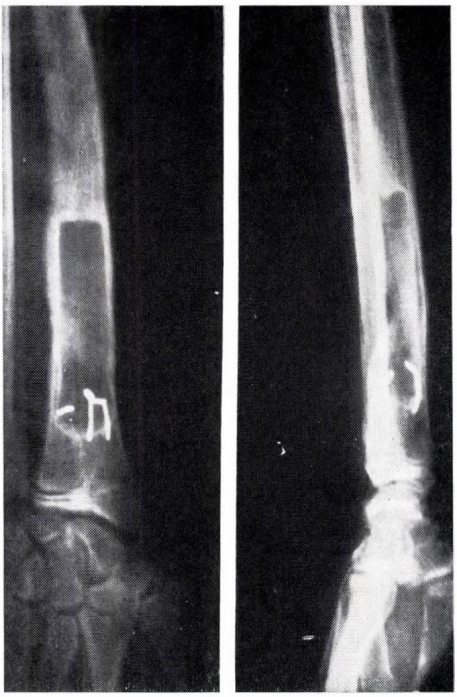


FIG. 13. Condition after removal of Küntscher nail used for internal fixation

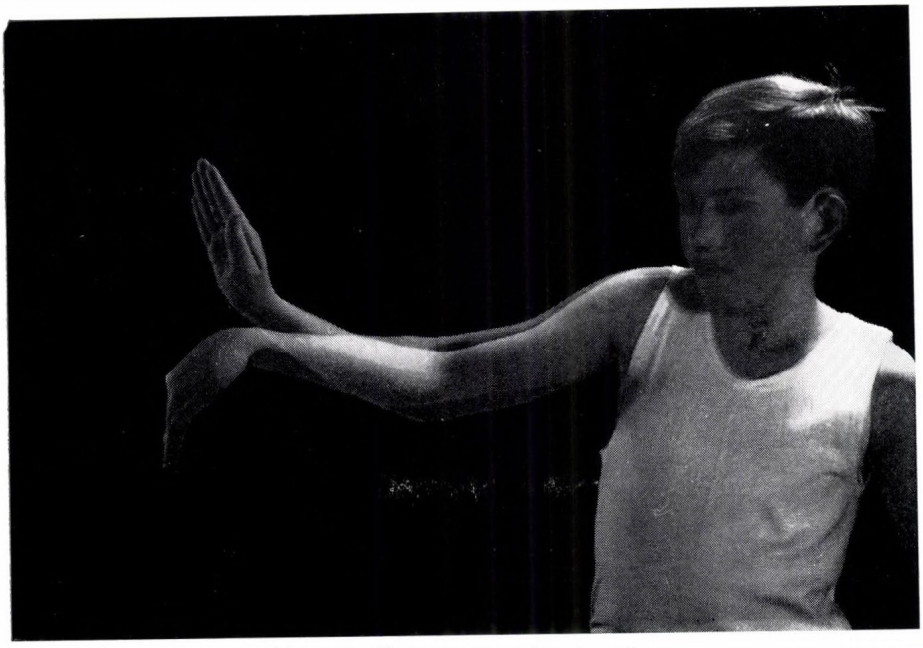


FIG. 14. Very good wrist function

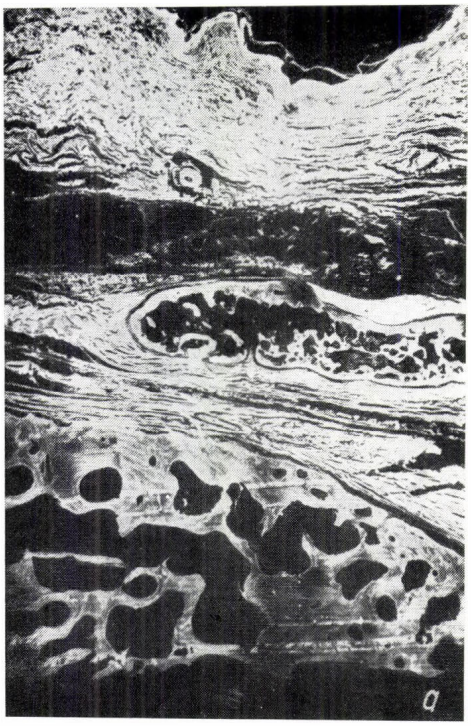


FIG. 15a. Histological picture of the reaction-free callus tissue, taken from the vicinity of the acrylate

FIG. 15b. Histological picture of the reaction-free callus tissue, taken from the vicinity of the acrylate

SUMMARY

Acrylate prosthesis has been employed to fill defects which resulted after extirpation of bone tumours. The procedure resulted in the formation of a fully developed bone tissue in the vicinity of the acrylate giving more stability of this graft. No recurrence or malignant degeneration was encountered.

According to the good results obtained with acrylate prosthesis, the use of this alloplastic is recommended in cases of filling bone defects.

Symp. Biol. Hung., 7, pp. 351—359 (1967)

SOME INVESTIGATIONS CONCERNING THE HEALING OF BONE FRACTURES TREATED BY CORTICAL FIXATION

by

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Cortical fixation is a percutaneous method. Employing it, the fixation is performed with special compression nails which clutch the surface of the bone for the purpose of pressing the surfaces of the fracture together. During compression, the unevenness of the fractured surfaces satisfactorily hinders any slips (Fig. 1).

With cortical fixation effective immobilization andan absolute reposition can be attained. We should like to demonstrate briefly their application in some cases.

At the fracture of larger bones the application and temporary fixation of the nails can be attained by employing another kind of compression apparatus (Figs 2 and 3). On the heads of the nails there are broad screw plates which will be fastened by the plaster for the purpose of final fixation. After the drying of the plaster bandage, the compressor is removed from the nails.

In the case of phalanx fracture, the cortical fixation can be carried out with a simple compression clamp (Fig. 4).

The illustrations demonstrate that after a satisfactory application of cortical fixation, consolidation ensues practically without a trace. The period of consolidation is the same as that after a fracture without dislocation of a simple crack. The functional healing of the extremity sometimes occurs more rapidly after cortical fixation than in the case of fractures without dislocation; presumably because after cortical fixation it is not necessary to employ the plaster bandage for such a long time or to such a great extent.

The importance of functional exercises cannot be underrated. Such exercises employed prudentially in the treatment of fractures fixed cortically are just as important healing factors as the perfect adaptation itself. It may occur that functional healing is protracted not only after cortical fixation but also after the use of other methods. The contractures dissolve

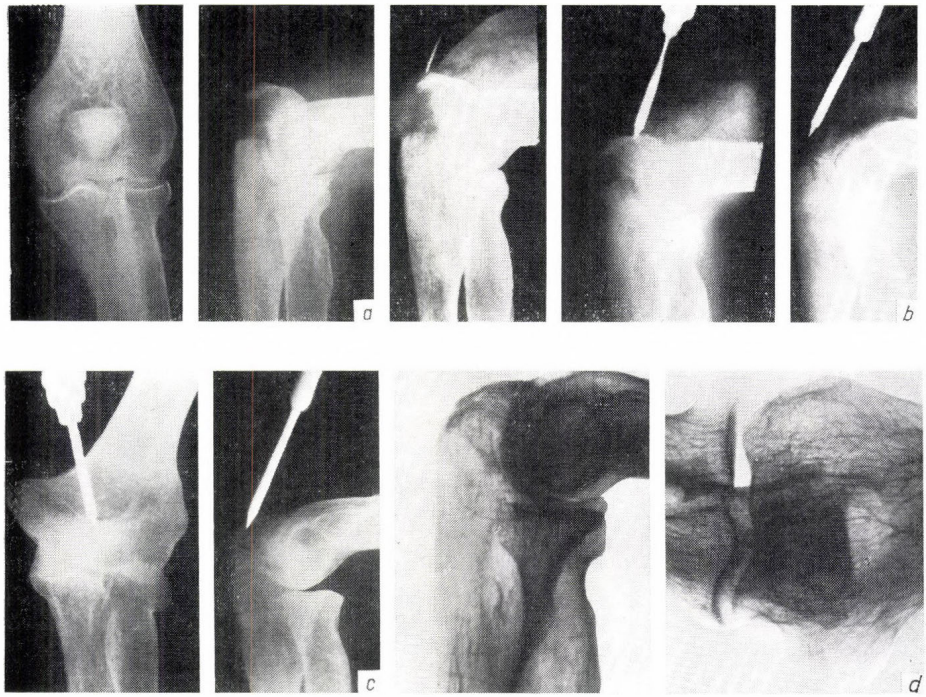


FIG. 1. Complete olecranon one-piece fracture before (a), during (b), and after (c) cortical fixation; 6 months after treatment (d) the structure of the bone has been completely re-organized. No signs of callus formation

with difficulty and the swelling disappears more slowly. It was conspicuous that in such cases the extent of the callus formation was greater every time than expected. This extensive callus formation was very rarely preceded by an extensive fracture haematoma. With regard to contractures, the delay of operation caused protraction of functional healing if also strong haemorrhage was present. But this prolonged functional healing occurred almost in every case when the patients were reluctant to perform the exercises or complained on account of them. Such complaints were generally voiced during the first days of the injury or operation, while the other extreme was to overdo the required exercises. In the latter case sometimes a loosening of the fixation ensued. But in every case leading to prolonged functional healing there was an unusually enlarged callus formation whether adaptation or fixation deteriorated or not.

Because of the fact that the larger callus is in connection with the undesirable exercises in the early period, it may be presumed that the well-known dynamic stimuli play a role in its development. It may also be that local circulatory disturbances contribute to its appearance. Since there is no manifestation of insufficiency in adaptation of fixation in most cases, the extent of the callus is due to the dynamic stimulus which effects not

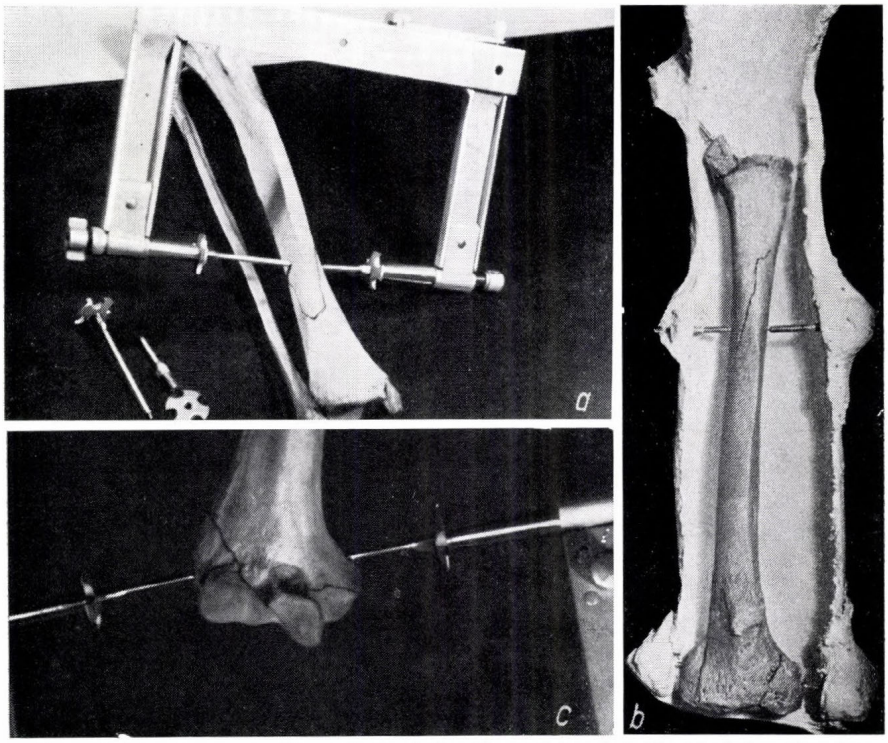


FIG. 2. Compressor with nails and fixation plates (a). Cortical fixation in fractures of the tibia (b). Y-shaped condylar fracture of the humerus fixed cortically (c)

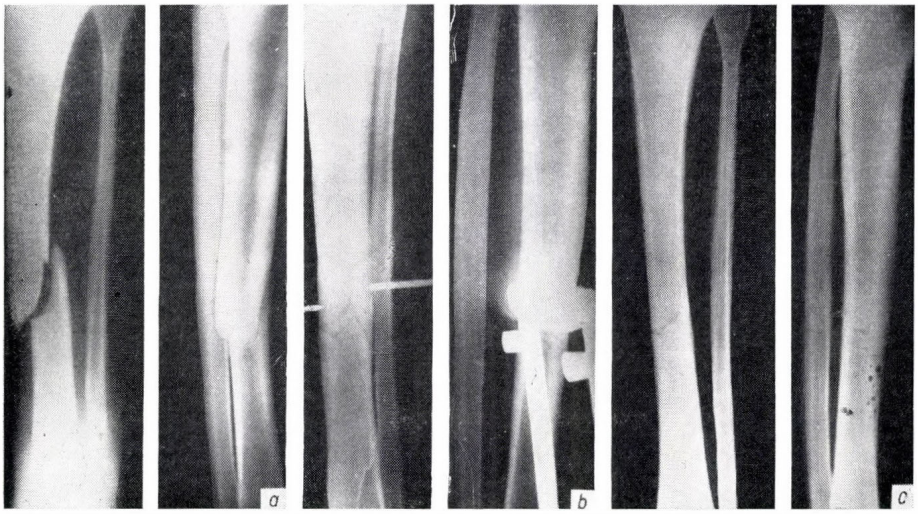


FIG. 3. X-ray picture of leg fracture before (a) cortical fixation, after operation (b) and after removal of the plaster (c); minimal callus formation

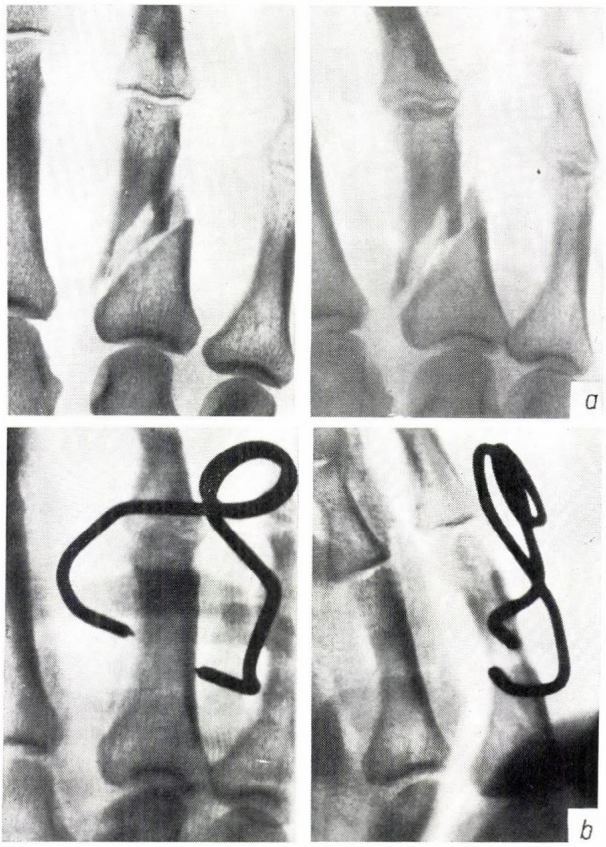


FIG. 4. Spiral phalanx fracture (a), after cortical fixation (b).

only the fractured bone but also the injured soft tissues. The question may be raised whether the enlarged callus is necessary concerning consolidation or not.

In case of healing with some slip of the fracture, the presence of the enlarged callus formation may undoubtedly be important, since the consolidation arises on account of its mass. In case of perfect adaptation, a smaller but qualitatively better callus elicits consolidation.

After the third or fourth week the exercises are not connected with similar callus formation or delayed functional healing. This warns us to pay attention not only to the fractured bone, but also to soft tissues surrounding it. The absorption of the fracture haematoma can be ensured well by the injured muscles and circulation only if they have overcome the lesion caused by the injury. For this reason these tissues also require care. Since the negative result of early functional exercises manifests itself not only as an undesirable callus formation but also as a protracted functional result, it must be assumed that this occurs because of the damage of the soft tissues.

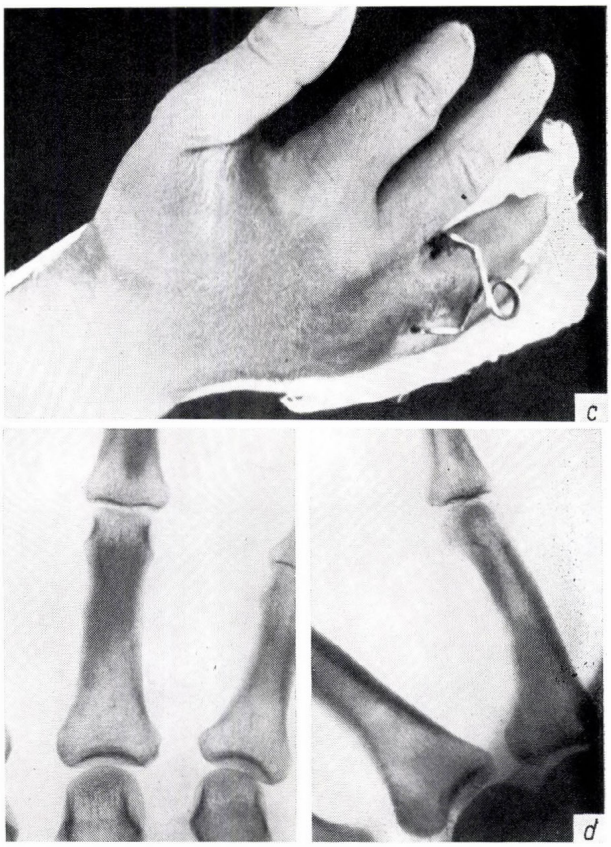
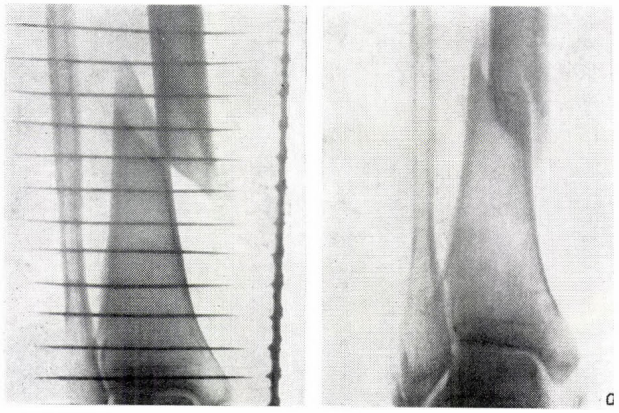
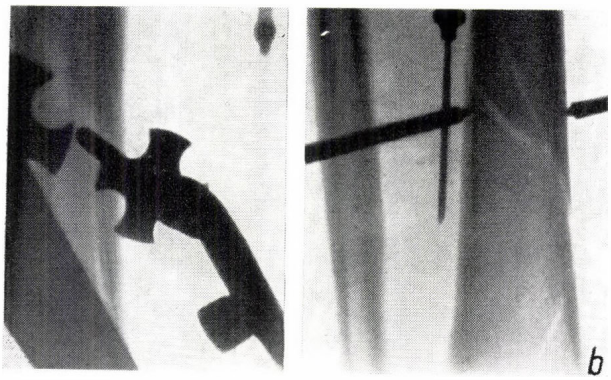


FIG. 4. The injured hand with the compression clamp on a protective splint (c). After consolidation without dislocation and callus (d)

On account of early disproportionate exercises, a secondary dislocation and an enlarged callus formation arise in very interesting correlation. The patient was admitted because of a leg fracture. Cortical fixation was carried out. At the time of the procedure the fissure was not detected and slipped apart during the exercises. Consequently, the previously replaced and fixed fracture part slipped apart, too. After this a massive callus developed in the thigh where the patient had suffered slight contusion. This massive callus was the result of forced exercises demanded by the functional treatment of the leg. After the fracture had been treated repeatedly with great care, it healed with a *relatively* smaller callus, but the functional healing was delayed (Fig. 5).

The above case proves that dynamic employment elicits callus not only in the bones but also in the soft tissues if they are damaged. In such a place there is no need of a callus. It is our task to promote the conditions for regeneration, i.e. reposition, fixation and to facilitate the circulation. In this respect the soft tissues should not be neglected either, because too early activization may be injurious.





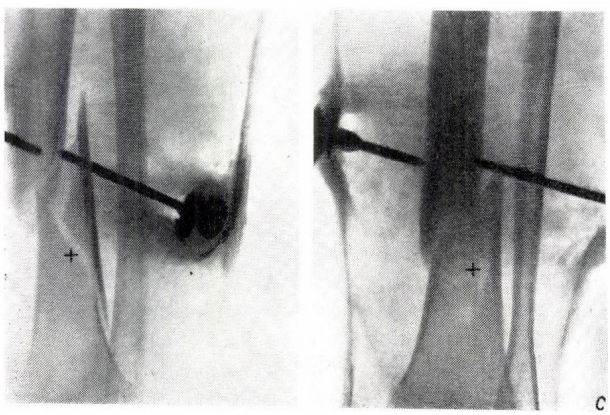


FIG. 5. Apparently simple spiral leg fracture (a), after the first treatment (b). The fracture slipped apart and the fracture line appearsed which had been invisible previously (c).

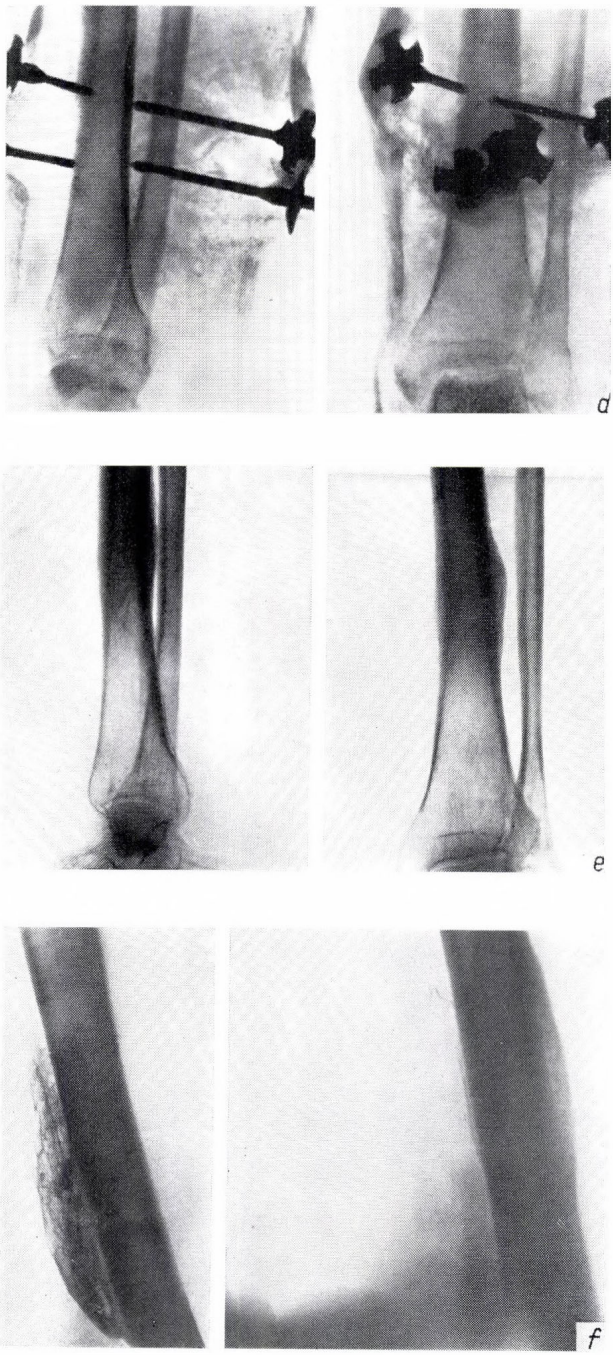


FIG. 5. After cortical fixation performed repeatedly (d). The fracture healed without dislocation with a comparatively small callus (e). An enormous callus formation in the muscle of the thigh without a fracture (f).

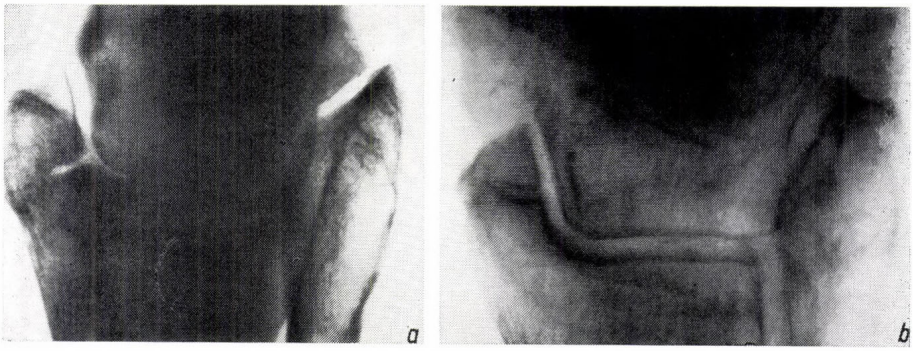


FIG. 6. Partial union after inner fracture of the ankle (a). A similar union after inveterated fracture (b)

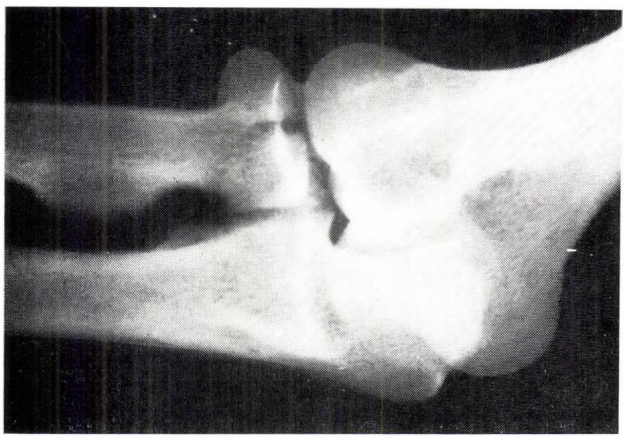


FIG. 7. A 2-year-old radius-head fracture with lack of bone union, treated in the usual way without notable effect

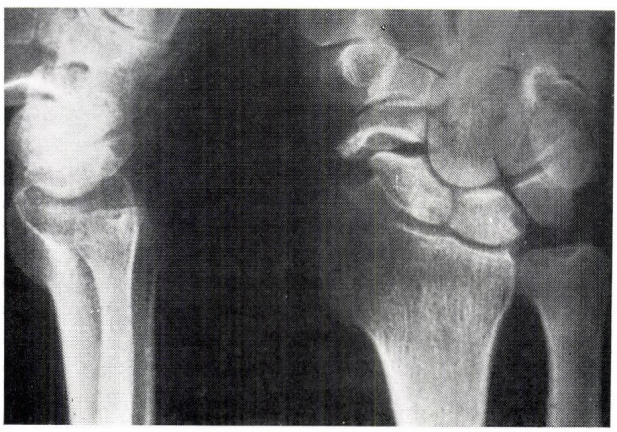


FIG. 8. Pseudoarthrosis of the scaphoid without essential arthrosis or functional damage, arising from injury sustained 30 years previously

With regard to cortical fixation it was observed that joint fractures consolidated in every case, so that the supply of blood to the bone was not improved by fixation. This is the case of the scaphoid. Sometimes the bone union developed only partially owing to the fractured surfaces. In the case of inside fracture of the ankle, a pseudoarthrosis appears, usually because of periostal interposition (Fig. 6). After cortical fixation lack of consolidation was never observed in such cases. In a few cases the bone union developed only partially, but this lack of bone union *sometimes ensues in the inner part of the fracture where the interposition mentioned above could be expected to occur*, but only at the site of the joint. In the case of fracture or pseudoarthrosis of the scaphoid, a cyst formation occurs at the line of the fracture. A lack of union can often be observed in elbow-joint fractures, too (Fig. 7).

Such a pseudoarthrosis is not accompanied by the arthrosis of the joint (Fig. 8).

As a result of these findings it can be concluded that the synovia of the joint can inhibit consolidation. This inhibitory substance may prevent arthrosis, but also promote the development of pseudoarthrosis. On account of the compression, however, this substance cannot affect the fractured surfaces, so that pseudoarthrosis cannot ensue after cortical fixation is applied perfectly. Perhaps the unfavourable healing tendency is a result of the development of pseudoarthrosis, or a renewed rupture of the knee-cap and olecranon, in fact, of the appearance of necrosis of the femoral head as well as of the femoral neck.

Because of the nature of its properties it may be worth while to clarify its biological effects, in fact, to isolate it for the purpose of treating arthrosis with its aid.

The findings mentioned above are important observations obtained in cortical fixation but, apart from these facts, cortical fixation solves other problems, too, which are connected with fractures. In the first place, open and inflamed fractures present difficulties which cortical fixation can overcome. In this case healing can be attained by improving the circulation both of the bone and of the soft tissues.

EFFECT OF ATP ON CHONDRAL BONE FORMATION

by

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See: *Morphologiai és Igazságügyi Orvosi Szemle* **6**, 36, 1966 (in Hungarian)

Symp. Biol. Hung., 7, pp. 361—362 (1967)

EFFECT OF DRUGS INFLUENCING VASCULARIZATION ON CALLUS FORMATION

by

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NUMEROUS experimental data (Ladányi and Hidvégi 1954, Trueta and Cavadias 1955) and clinical experience (Harris and Hobson 1956, Hellstadius 1950, Hiertonn 1957, etc.) indicated that a close correlation exists between fracture healing and vascularization. According to Krompecher (1937, 1956), chondral ossification is attributed to vascular pauperization ensued under the influence of mechanical stresses (compression).

In the present experiments the effect of local administration of vasoactive substances on the processes of callus formation was investigated.

The experiments were carried out on 144 albino rats. The left femora of the rats were fractured and the bone ends were fixed by a special cannula which, besides fixation, permitted prolonged local administration of drugs. One group of rats were given 0·1 mg/rat *noradrenalin*, a second group received 5·0 mg/rat *acetylcholine* and a third group was treated with 5·0 mg/rat *histamine*, in daily doses. The fourth group served as control. The rats were killed after 7, 10, 14, 17, 21 and 28 days of treatment. The removed callus specimens have been examined by histological and histochemical methods.

Some rats were injected with India ink prior to killing.

In the 7-day-stage after fracture, the ingrowth of vessels from the periosteum was inhibited owing to the effect of noradrenalin. Consequently, a large cartilage islet containing acid mucopolysaccharides appeared. In the groups treated with acetylcholine and histamine there was a marked ingrowth of capillaries. The vessels were tortuous with wide lumina. The histological sections showed that in these cases the callus consisted of a denser connective tissular granulation tissue. Cartilage formation was strikingly inhibited (Fig. 1). Fourteen days after fracture the vasoconstriction and, parallel to it, the extent of the cartilage islet showed a further increase. Capillarization increased due to the effect of acetylcholine and histamine, though the ingrowth of the vessels was not uniform. Cartilage appeared in the vicinity of the fractured ends, at sites where the vessels were absent. The extent of this cartilage is even smaller than that of the controls. In the acetylcholine-treated rats, and partly in those treated with histamine, ossification proceeded rapidly. In the 28-day-stage the vascularization of the callus—due to noradrenalin treatment—was found to be poor compared with that of the controls or acetylcholine-treated rats. Consequently, the amount of persisting cartilage is considerable. The calluses in the controls,

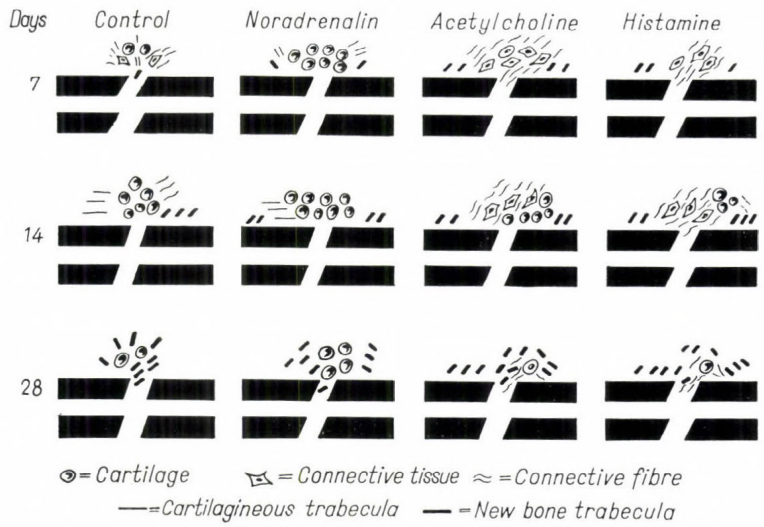

FIG. 1

and especially in the rats treated with acetylcholine and histamine consist mostly of new spongious bone.

According to these experimental results, local noradrenalin treatment has an inhibiting effect on the vascularization of callus by which the extent of cartilaginous callus is increased and fracture healing is retarded. Acetylcholine and histamine treatment resulted in an improvement of vascularization of the callus by which only a minimal cartilaginous callus is produced and at the same time ossification was increased.

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Symp. Biol. Hung., 7, pp. 363—364 (1967)

EFFECT OF PRESSURE ON CALLUS FORMATION

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THE ROLE of mechanical factors in ossification has been investigated by numerous authors, especially since Krompecher's experimental results (1937, 1943). The investigations of Oberdahlhoff (1948), Yamagishi and Yoshimura (1956) and others confirmed the role of pressure in cartilage formation, while for other authors the role of pressure in cartilage induction is questionable (Matzen 1955, Karlinger 1957).

The purpose of our investigations was to study the role of pressure in callus formation. The experiments were carried out on 24 dogs. A V-shaped fracture was produced on the tibia of the dog. The fractured ends were fixed by a transversal wire and a pressure of 8 kg was applied on them by a device provided with a spring. The stages of 10, 14, 21, 28, 35 and 42 days were investigated by histological and histochemical methods. These stages are schematically represented in Fig. 1.

In the 10-day-stage the broken bone ends were found to be in complete apposition with a minimal haematoma between them. In the developing new spongious bone substance of the external callus connective tissue was visible.

In the 14-day-stage the haematoma was still visible between the fractured ends. Cartilage made its appearance in the external callus. The internal callus consisted of granulation tissue.

In the 21-day-stage cartilage appeared in the internal callus as well. At the fractured ends necrotic bone and slight amount of connective tissue was demonstrable.

In the 42-day-stage both the external and internal callus consisted of young spongious bone substance.

By histochemical reactions, acid mucopolysaccharides were demonstrated in accordance with the extent of cartilage.

According to our results, the granulation tissue is gradually replaced by cartilage, first in the external then in the internal callus. In later stages the cartilage is substituted by a new spongious bone substance. Under the present experimental conditions no cartilage formation was observed between the fractured compact bone ends.

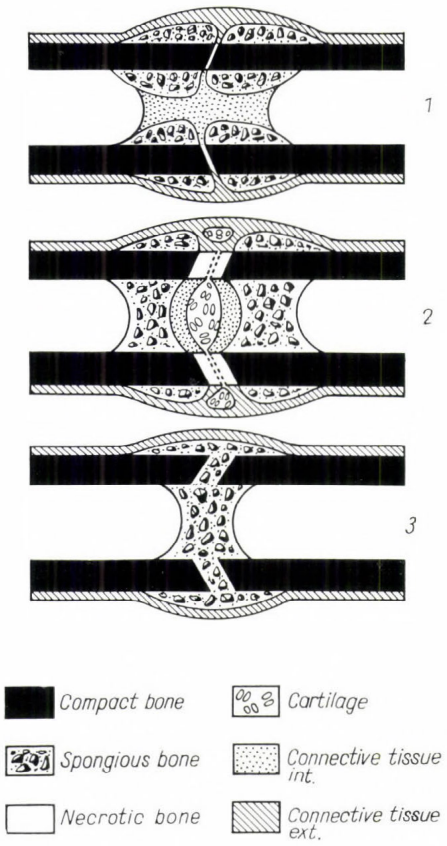

FIG. 1. Schematic illustration of the different stages of callus formation under the influence of pressure; 1 = approximately 10 days after fracture: compression forces are transmitted by the compacta; no cartilage (no preformative tissue), 2 = approximately 2 to 4 weeks: compression forces are transmitted by spongious bone trabeculae; cartilage has appeared (preformative tissue exists); the broken bone ends are necrotized; 3 = approximately 6 weeks: compression forces are transmitted by completely developed new bone trabeculae; the cartilage islets are ossified

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HEALING OF ARTICULAR CARTILAGE FOLLOWING ARTIFICIAL INJURIES

by

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See: *Magyar Traumatológia, Orthopaedia és Helyreállító Sebészet* **8**, 128, 1965 (in Hungarian)

FATE OF FUNCTIONLESS AUTOGENOUS AND HOMOGENEOUS GRAFTS IN THE ORGANISM

by

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BEHAVIOUR OF THE REGENERATING ARTICULAR SURFACE IN THE ANTERIOR CHAMBER OF THE EYE

by

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See: *Morphológiai és Igazságügyi Orvosi Szemle* **5**, 36, 1965 (in Hungarian)

Symp. Biol. Hung., 7, pp. 367—370 (1967)

PHOTOELASTIC INVESTIGATIONS ON DIFFERENT GELATIN SAMPLES

by

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IN THE course of the causal analysis of the formation of newly differentiated cartilage, the question arose whether the stress caused by compression is necessarily identical or it may be different at different levels of the granulation tissue layer (1000 to 1500 μ thick) covering the injured bone surface facing the articular cavity.

On the basis of his preparations demonstrating vascular constriction and occlusion increasing in each layer, as well as of the consistent appearance of the cartilage islet in the lowest layer, Krompecher (1956) came to the conclusion that the stress must be different in different depths of the granulation tissue. On the contrary, Pauwels (1960) claimed, referring to rules of hydromechanics, that the stress should be identical everywhere in the granulation tissue. Krompecher and Tóth (1964, 1965) demonstrated in experiments on a gelatin model, containing a capillary network (rubber tubes), that the stress acting on a circumscribed area spreads with decreasing intensity on account of the elasticity of the medium. Pascal's law does not hold for the granulation tissue, since it is not a fluid and is not contained in a closed system.

In the course of the discussion arose the necessity of performing photoelastic examinations on similar models, as the above problem can also be decided experimentally by using photoelastic methods. The essence of the photoelastic method is to investigate stress distributions in an optical way. The basis of the method is the fact that a body isotropic for polarized light becomes anisotropic if subjected to mechanical stresses. The so-called photoelastic picture appearing in polarized light depends on the stress distribution induced by outer forces. The density of a transparent body subjected to mechanical load will be different in the directions of the different principal stresses, so the velocity of the polarized light will also differ, the entering polarized beam will be split into components of the directions of the principal stresses. So a phase difference arises between the components of the emerging light vector, and the photoelastic picture appears as a result of the interference between these components. This picture consists of coloured lines or bands and black lines or points in the case of white light, while it consists of black lines or bands, black points in the case of monochromatic light (Coker and Filon 1931).

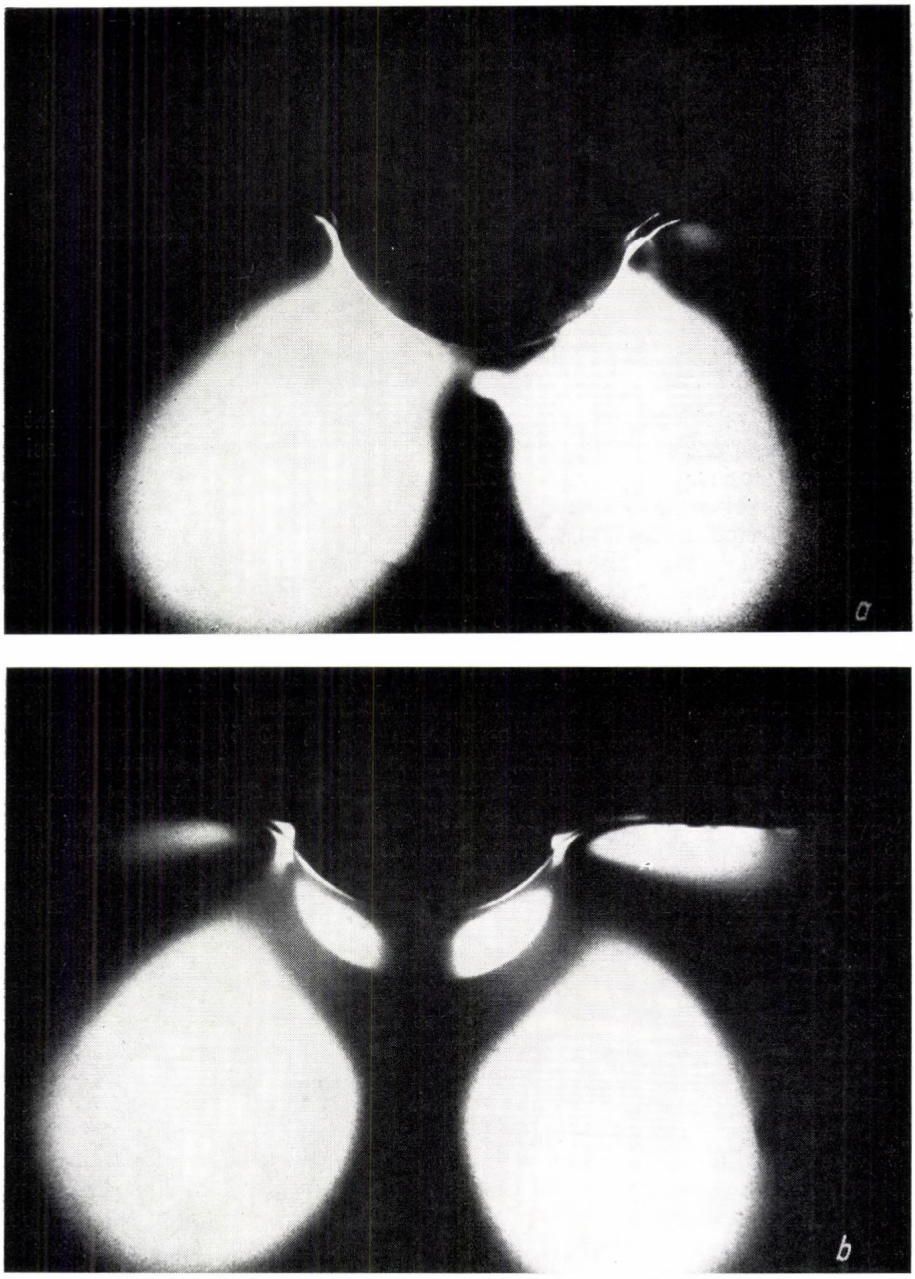
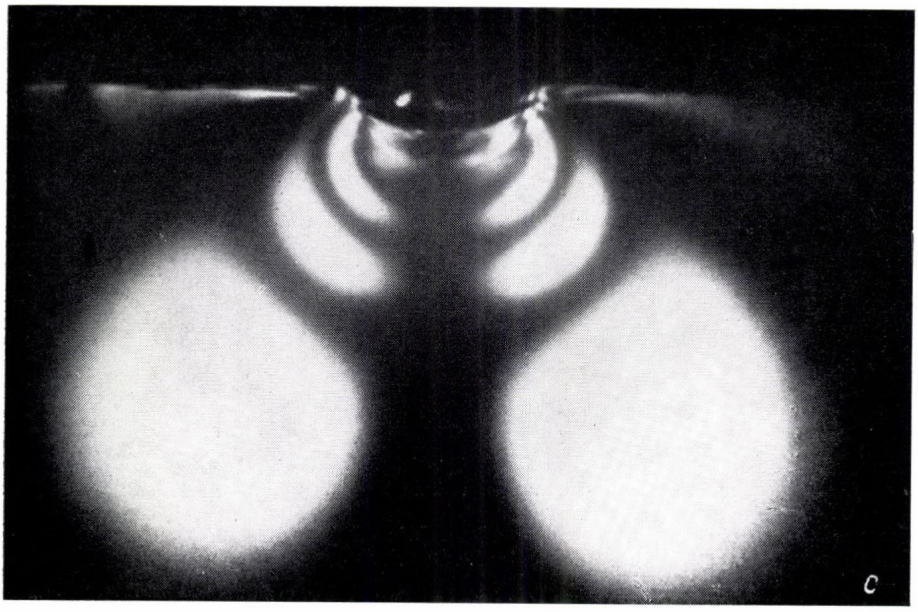


FIG. 1. The photoelastic pictures show different distributions of stress at different depths of the samples; a = 2·5% gelatin solution. Temperature: 22 ± 1 °C. Loading about 50 g; b = 5% gelatin solution. Temperature 22 ±1 °C. Loading about 50 g;



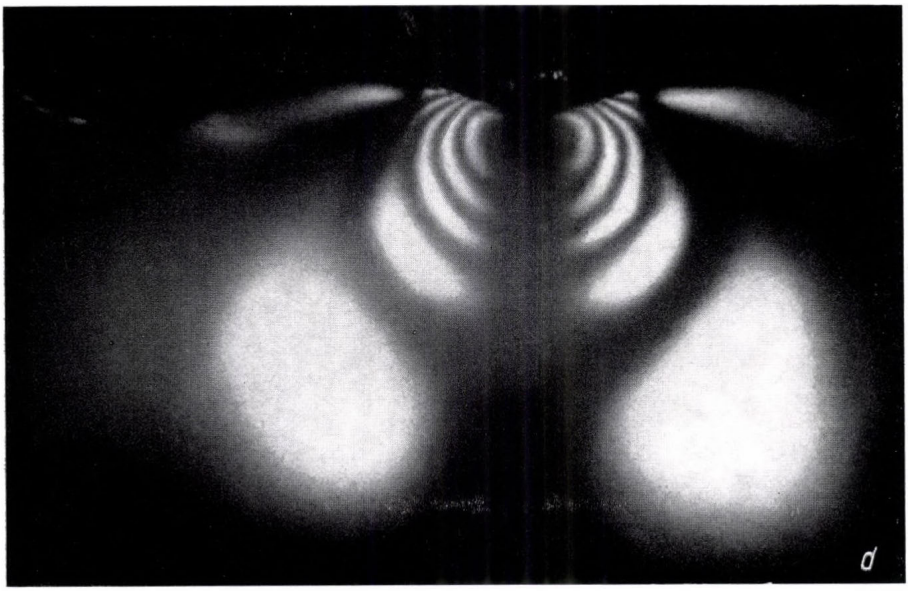


FIG. 1. c = 10% gelatin solution. Temperature: 22 ± 1 °C. Loading about 250 g; d = 20% gelatin solution. Temperature: 25 ±1 °C. Loading about 250 g

In order to decide the problem in question, it is not necessary to treat it quantitatively, although it is not difficult in simple cases (Jessop and Harris 1960). If a loaded sample, approaching more or less the real situation, exhibits photoelastic picture through crossed polarizator and analizator in polarized light, Pascal's law does not hold for such systems (if it does, the strain distribution is isotropic!)

The models were prepared of gelatin-water solution containing 2·5 to 20% gelatin. The solution was poured into a mould made from plexi glass. The mould was not removed even after solidifying, so the stresses induced by the weight of the gelatin could be avoided. The investigations have been performed at 22 ± 1°C, and 25 ± 1°C for different loadings. In every case a photoelastic picture appeared, (Fig. 1) which clearly shows that Pascal's law is not valid for such systems.

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PROMOTION OF VASCULARIZATION OF BRADYTROPHIC TISSUES EXPLANTED TO CHORIOALLANTOIC MEMBRANE

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See: *Anat. Anz. Suppl.* **109**, 126, 1962

Symp. Biol. Hung., 7, pp. 371—373 (1967)

OSTEOGENESIS IN VERTEBRAE OF THE RAT TAIL INDUCED BY LOCAL ADMINISTRATION OF ADRENAL EXTRACT

by

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The conditions of osteogenesis have been extensively treated in the literature. Krompecher (1940, 1958) emphasized the role of certain hormonal and biochemical factors, the presence of undifferentiated mesenchymal cells and capillarization as important requirements of osteogenesis. Adrenal extract prepared from homogenates of suprarenal glands of adult rats was found (Kiss and Krompecher 1962, Krompecher and Kiss 1962) to be most effective in promoting vascularization by which osteogenesis was likewise stimulated. Local administration of adrenal extract was found to induce primary angiogenic bone formation (Kiss 1964, Krompecher and Kiss 1963).

Experiments were conducted on 14 adult albino rats of both sexes. Under ether anesthesia a canal was formed by a sterile flexible wire drill through

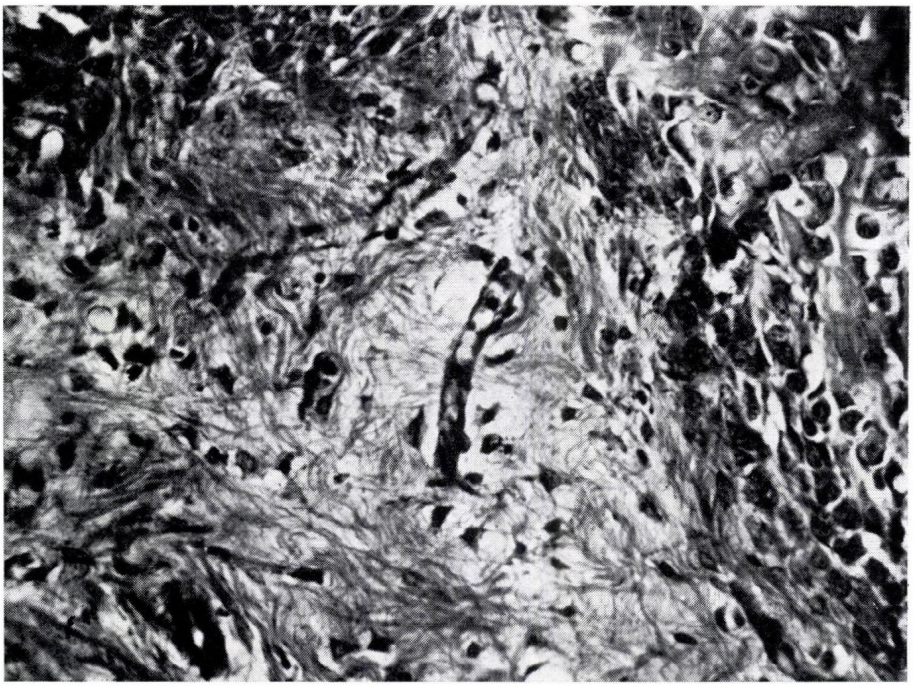

Fig. 1. Multiplication of capillaries and osteoblasts in the periosteum of rat tail vertebra following local injection of adrenal extract (1-week-stage)

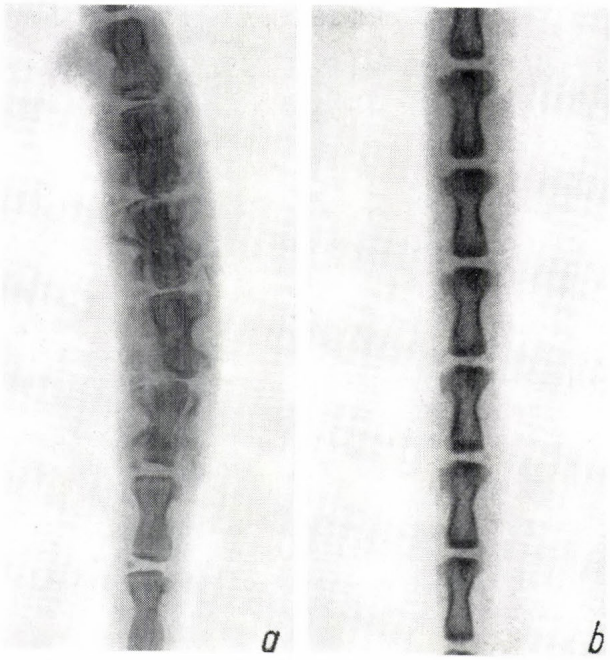

FIG. 2. Positive X-ray picture of rat tail vertebrae 4 weeks following injection of adrenal extract (a); X-ray picture of control (b).

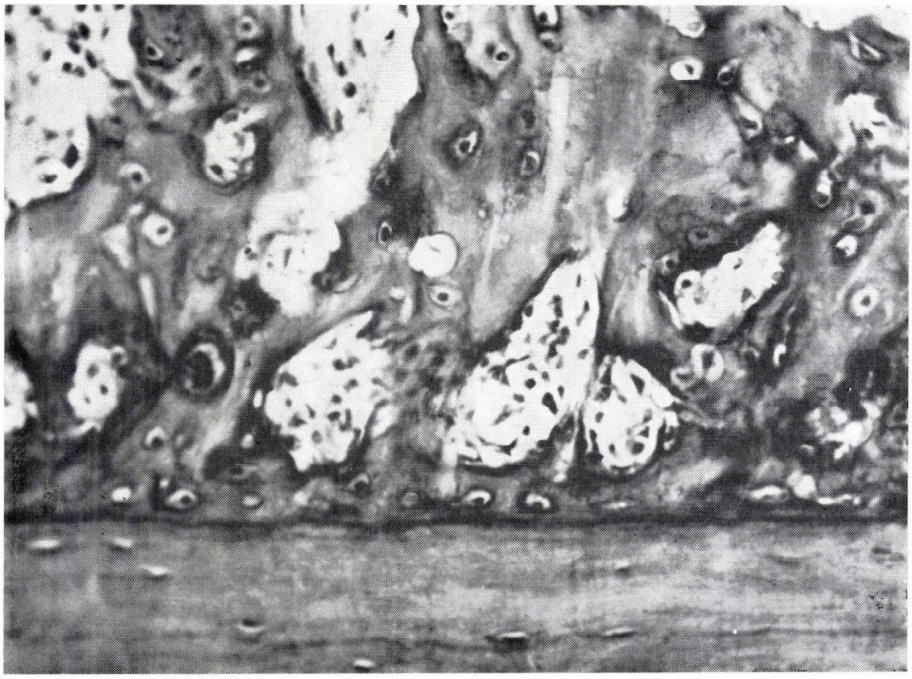

FIG. 3. Under the effect of adrenal extract new bone with red bone marrow was formed in connection with the old bone (4-week-stage)

the vertebral bodies of 4 to 5 caudal vertebrae. Adrenal extract, prepared from rat and human suprarenal glands, was injected in a single dose in the canal prepared in this manner. The rats were sacrificed after 1, 2, 3 and 4 weeks and 6 months, respectively. The material removed was examined by histological methods.

One week after injection of adrenal extract, a multiplication of the capillaries and osteoblasts of the periosteum was observed in the histological preparation (Fig. 1).

In the second, third and fourth week, a gradually increasing subperiosteal bone formation was demonstrated which was evidenced by radiography as well (Figs 2 and 3).

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Symp. Biol. Hung., 7, pp. 375—377 (1967)

EFFECT OF CHRONIC ALCOHOL TREATMENT ON BONE REGENERATION

by

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THE FACTORS influencing the healing of bone fractures have been extensively investigated. Numerous researchers dealt with and emphasized the importance of the mechanical factors (Friedenberg and French 1952, Krompecher 1937, Yamagishi and Yoshimura 1955), and that of the capillary supply depending on them. Numerous investigations have been carried out on the effect of chemical factors, too (Burkhardt 1928, Levander 1938).

The purpose of our experiments was to investigate the effect of alcohol treatment on fracture healing, considering that the alcohol has a peripheral vasodilatating effect and it increases the oxigenization of blood.

Albino rats of both sexes were used. The femora of the rats were fractured and fixed by percutaneous medullary nailing. A group of rats were given, through a gastric tube, a daily amount of 1·6 to 4·26 g/kg body weight alcohol per rat. The controls received water and a third group was treated with powdered egg-shell (Lelkes and Mészáros 1957). The rats were killed 1, 3 and 5 weeks following operation. The results obtained are shown in Figs 2 to 4, and explanatory key is given in Fig. 1.

After *one week* of treatment, granulation tissue, cartilage and angiogenic osteophytes were found between the fractured bone ends. The smallest amount of cartilage was found in the alcohol-treated group, while the largest amount and most mature cartilage was demonstrated in the egg-shell-treated group. Somewhat more osteoblasts were seen in the alcohol-treated group (Fig. 2).

In the *3-week-stage* more capillaries were found in the remnants of granulation tissue in the alcohol-treated groups than in the controls. In the axis of the newly formed bone trabeculae the remnants of the ossified cartilage were found in a broader zone in the alcohol-treated group than in the controls. The amount of cartilage increased in all three groups displaying the same distribution as in the first week (Fig. 3).

In the *5-week-stage* bone trabeculae were predominantly present between the fractured ends but while in the controls cartilaginous and even granulation tissue elements were found, complete fusion of the bone margins was observed in the alcohol-treated group in the majority of the cases (Fig. 4).

It may be concluded that the capillarization of the granulation tissue increased and, parallel to it, the formation of bradytrophic cartilage tissue

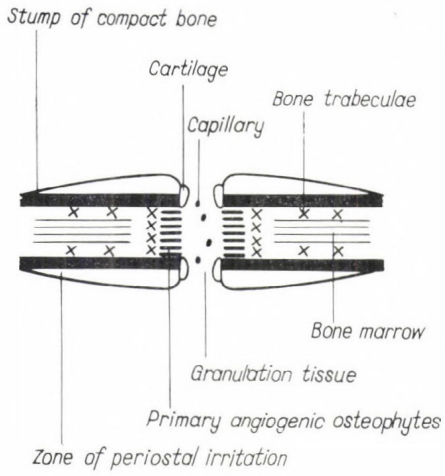

FIG. 1

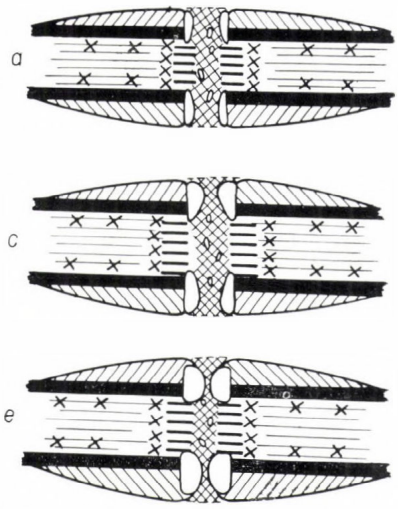

FIG. 2. Results obtained after 1 week; a = alcohol treatment; c = control; e = egg-shell treatment

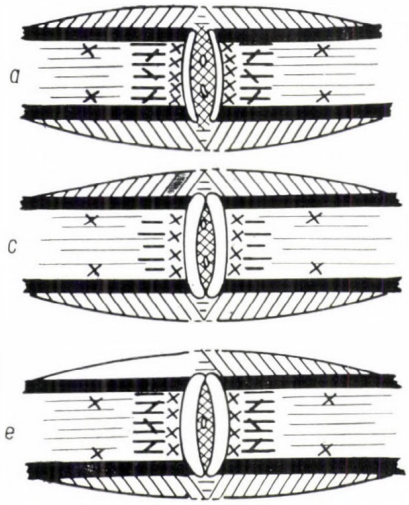

FIG. 3. Results obtained after 3 weeks; a = alcohol treatment; c = control; e = egg-shell treatment

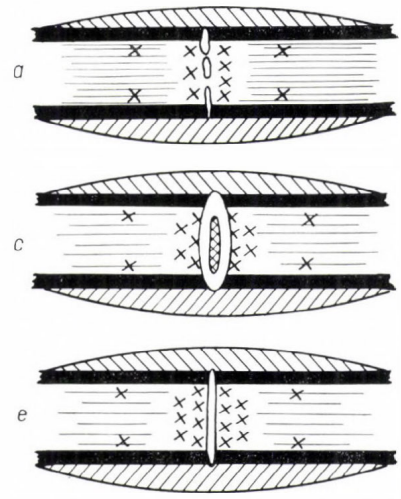

FIG. 4. Results obtained after 5 weeks; a = alcohol treatment; c = control; e = egg-shell treatment

was somewhat inhibited in the alcohol-treated rats. At the same time, ossification seemed to be accelerated in these cases as evidenced by the young cartilage cells present in the interior of the newly formed bone

trabeculae. In this effect of alcohol, certain substances formed in the course of its break-down (fumaric acid, succinic acid, etc.) may also play a part by their catalytical increase of tissue respiration. Further investigations are in progress.

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Symp. Biol. Hung., 7, pp. 379—382 (1967)

PROMOTION OF THE PROCESS OF ORGANIZATION OF THE KIEL BONE GRAFT

by

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IN AN EARLIER report (Kondrai and Tarsoly 1965) we reported our experiments on the use of the Kiel bone graft described by Bauermeister (1958) and modified by Kondrai (1964). The results obtained in these experiments have demonstrated that cancellous bone grafts of heterologous origin become fairly organized and had a stimulating effect on osteogenesis. The organization of compact bone grafts was found to be worse; they even failed to organize if taken from older animals by exerting an inhibitory effect on osteogenesis. Such bone grafts were soon resorbed and rejected by the host organism. For these reasons we have made attempts to increase the inclination for organization of the compact bone graft by various pretreatments. It is known that the smaller the bulk of the implanted bone, the better it survives since it gets into touch with larger areas of the recipient host. Therefore, various bone preparations were made:

1. compact bone graft ($2 \times 0{\cdot}5 \times 0{\cdot}5$ cm) was prepared for control;
2. on all sides of a compact bone graft, the same size as the previous one, small holes were bored;
3. the compact bone graft was excised together with its natural spongiosa covering one of its sides;
4. on the smooth sides of the compact bone, prepared as previously, some holes were bored (Kondrai and Tarsoly 1965);
5. on the smooth side of the preparation (see under 4), a layer of spongious bone was stuck; in this way, two surfaces facing each other were covered with spongious bone;
6. finally, the smooth surfaces of two bone grafts (see under 3) were stuck together.

The cross-sections of these experimental bone grafts were square as opposed to those employed by Maatz which were cylindrical. As to the further steps, we proceeded according to the experiments of Maatz on the spongiosa test, implanting the prepared bone grafts into spongious bone substance of dogs. Six implantations were performed with each type of graft (36 operations), implanting six different grafts in the same dog. Comparable results could be obtained by this method. The dogs were

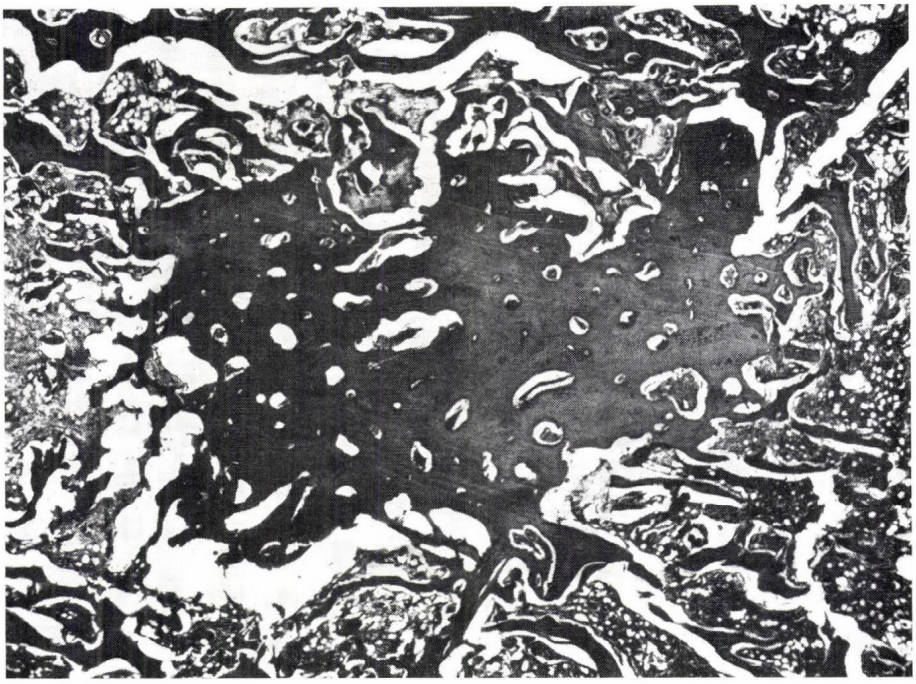

FIG. 1. Advanced organization of a compact bone graft with borings on one of its sides and natural spongious layer on another side 4 weeks after operation

sacrificed after two and four weeks following operation. The implanted area was excised and studied by histological methods. For the sticking together of the bones we experimented with 16 kinds of glues, of which the synthetic material called polyvinilacetate seemed to be the most suitable. In order to retard the quick evaporation of the solvent, dibutilphtalate was added to it which, however, had a damaging effect on osteogenesis as it was found out later, and even sticking itself was not of the best kind. The following results were obtained from the histological preparations.

The bone grafts with surfaces enlarged by borings were found to be organized fairly soon, since by the connective tissue growing into the holes and by the new bone trabeculae formed later the grafts become fixed. Considerable organization of the graft was noted after four weeks.

Still better result was observed in the graft whose surface had been enlarged on one side by a layer of natural spongiosa and on another side by borings. The spongious part was rapidly organized; on the surface of the grafts new bone was formed by which the implant was firmly fixed and organization began on all its sides. After four weeks the graft had considerably decreased in bulk, organized and surrounded by newly formed bone trabeculae (Fig. 1).

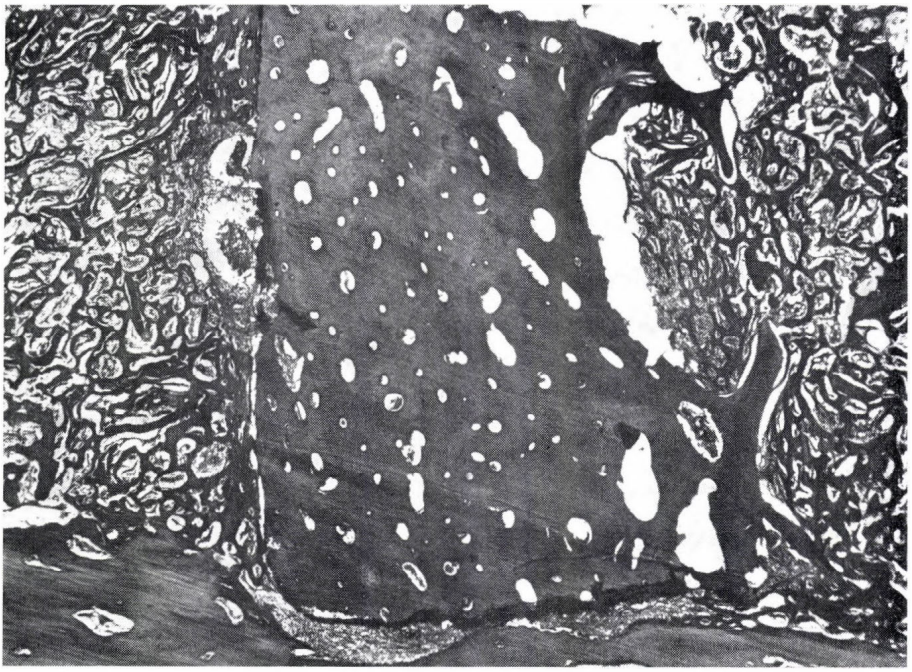

FIG. 2. Organization of a compact bone graft covered on one side by a natural spongious layer and by a spongious layer adhered to its other side 4 weeks after operation. Resorption and new organization are satisfactory. In the adhered part the spongious bone alone was found to be organized, but not the sticking material

The compact bone graft with a natural spongious bone layer on one side and with a spongious bone layer stuck to its other side, was also found to organize, but the adhered surface resisted organization, sharply delimiting the area of resorption and new formation of bone (Fig. 2). New bone trabeculae were formed in both spongious bone layers. In the compact bone graft consisting of two pieces adhered together, having a natural spongious bone layer on one side of both pieces, advanced organization was revealed starting from the spongiosa even after two weeks. After four weeks, considerable resorption and new organization were observed in the compact bone as well. Here the two parts detached usually displaying a narrow empty gap or filled up with connective tissue between the two bone grafts (Fig. 3).

According to our experiments, the organization of the cortical bone graft may be increased by enlarging its surface with borings or applying a spongious bone layer to it. In this way the ingrowth of vessels is facilitated which is a prerequisite of the onset of bone formation. However, further experiments are needed for the selection of a more adequate sticking material.

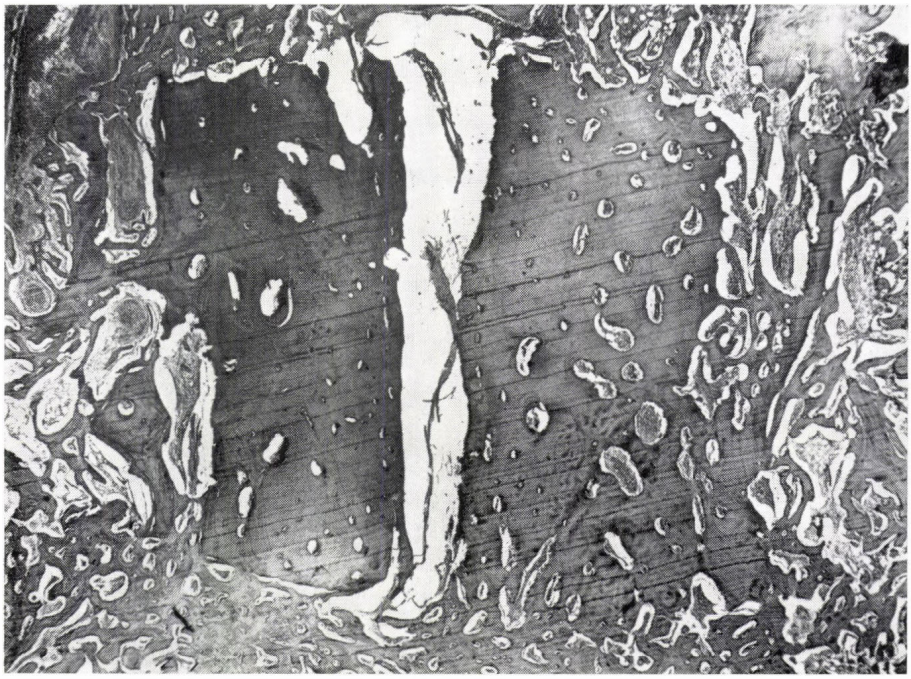

FIG. 3. Compact bone graft composed of 2 bone pieces stuck together, covered with a natural spongious bone layer on one side of both pieces, became considerably reduced in size due to new organization starting from the spongiosa. The 2 pieces detached owing to unsatisfactory sticking

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EFFECT OF PREDNISOLONE (DELTA-1-HYDROCORTISONE) ON THE NEODIFFERENTIATION OF CARTILAGE

by

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Symp. Biol. Hung. 7, pp. 383—387 (1967)

NEW FORMATION OF ARTICULATIONS

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ACCORDING to experimental results we are able to induce the organism to transform granulation tissue to articular hyaline cartilage. This may be of interest both to theoreticians and to clinicians.

As the process of formation of articular cartilage—including its causal motives—is very similar to that of cartilage formation in callus, it seems to be justified to demonstrate here some gross anatomical specimens and their microscopical preparations, by which the subsequent stages of experimental neodifferentiation of articular cartilage can be followed.

Experiments were carried out on dogs performing an operation on their knee-joints. The entire articular surface of the distal part of the femur was cut off together with a layer of the underlying cancellous bone. All cartilaginous remnants of the articular surface were likewise removed (Fig. 1). The source of the new cartilage was the granulation tissue taking its origin from the bone marrow. The granulation tissue soon covered the wound of the cancellous bone. To enhance the formation of a layer of granulation tissue, postoperative rest (five days) was ensured. Subsequently, an adequate functional treatment was instituted consisting of stretching and bending the limb in the knee-joint, twice every second day (Krompecher 1937, 1955, 1956, 1958a, b, 1966, Krompecher and Goerttler 1938). The granulation tissue was found to grow out from the red marrow covering the bone surface in a layer of 1·5 mm thickness (Fig. 2a). On the macroscopic specimen the sites of excisions are easily recognized. The microscopic picture demonstrates a healthy granulation tissue supplied with blood vessels. The surface is covered by a layer of fibrin (Fig. 2b).

Twenty-six days after operation (Fig. 3a) the new "articular" surface is smooth, though in the depth (left side of picture) the onlay of cartilaginous islets is visible. A cross-section of the specimen reveals (Fig. 3b) that in the depth of the vascularized granulation tissue, non-vascularized cartilaginous islets are already present.

Sixty-three days after operation the cartilaginous islets became manifest even on gross-anatomical inspection (Fig. 4a), though their surface was still covered by connective tissue containing some vessels (Fig. 4b). The cartilage islets showed a tendency to become confluent. As to its character, this cartilage appeared to be rather fibrotic.

Two-hundred and twenty-six days after operation (Fig. 5a) in a dog having a well functioning limb, larger cartilaginous islets separated by

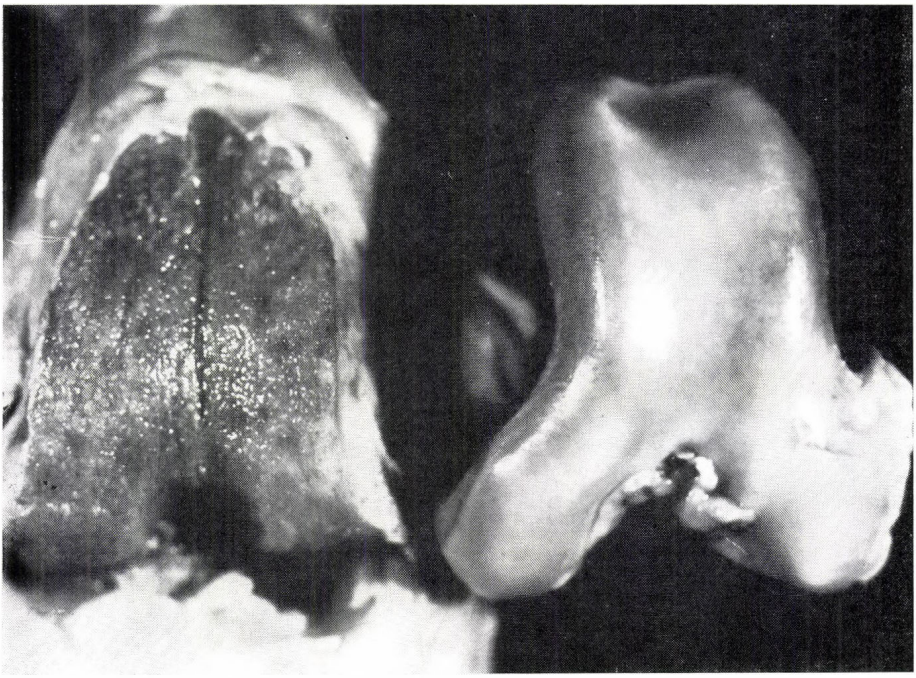

FIG. 1. Articular surface of the knee-joint was sawn off with a layer of underlying cancellous bone. On the left: the bone wound with its bloody surface. On the right: the articular surface that has been cut off

fossulae synoviales appeared. The microscopic examination (Fig. 5b) disclosed that the differentiation of granulation tissue to cartilage proceeded further and already reached the articular surface.

The articular cartilage was found to be fully developed in a 692-day-old specimen (Figs 6a and 6b) both gross anatomically and microscopically. Only a thorough comparison can reveal some differences, the newly formed articular surface having a deeper furrow and the condyli being more divergent than in the original specimen. The microscopical examination evidences a perfect articular hyaline cartilage.

This series of experiments carried out on dogs proves that the grown-up organism is able to differentiate to hyaline cartilage if adequate postoperative treatment is ensured. As regards further literature we refer to the papers of Krompecher and Hadházy published in this volume.

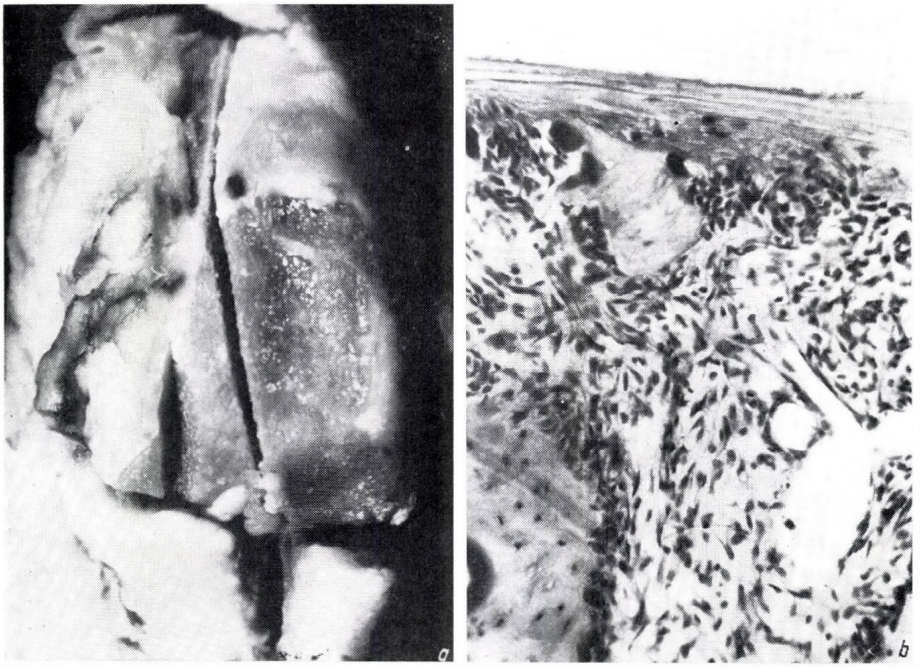

FIG. 2. a) 11 days after operation. b) Microscopic picture of 11-day-old specimen

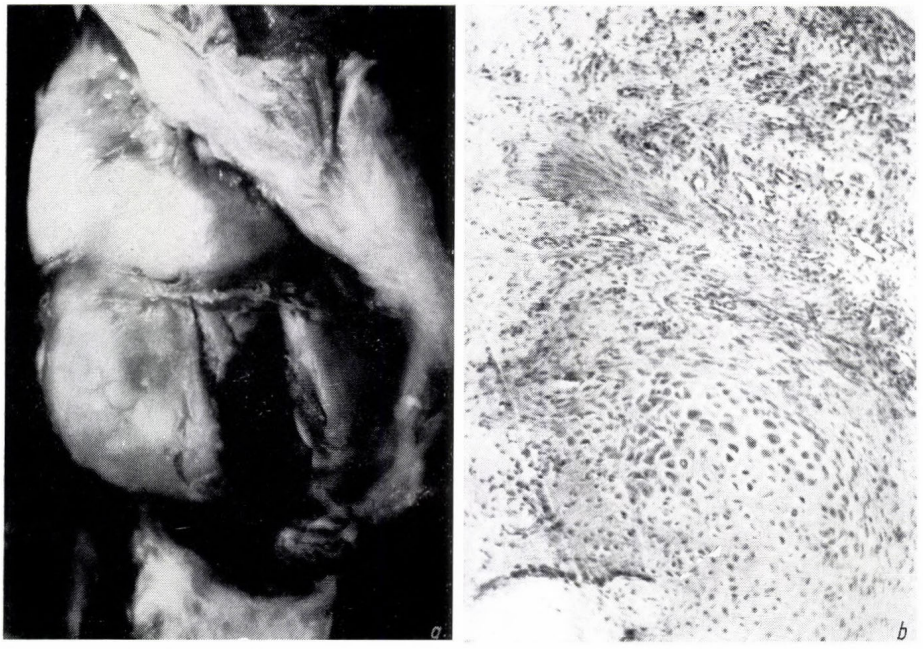

FIG. 3. a) 26 days after operation. b) Microscopic picture of 26-day-old specimen

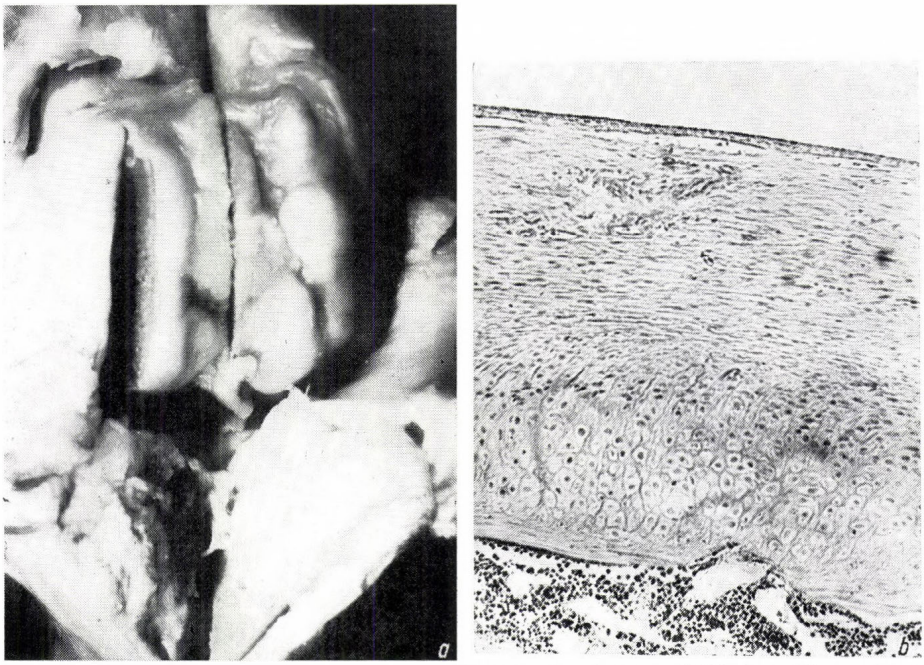

FIG. 4. a) 63 days after operation. b) Microscopic picture of 63-day-old specimen

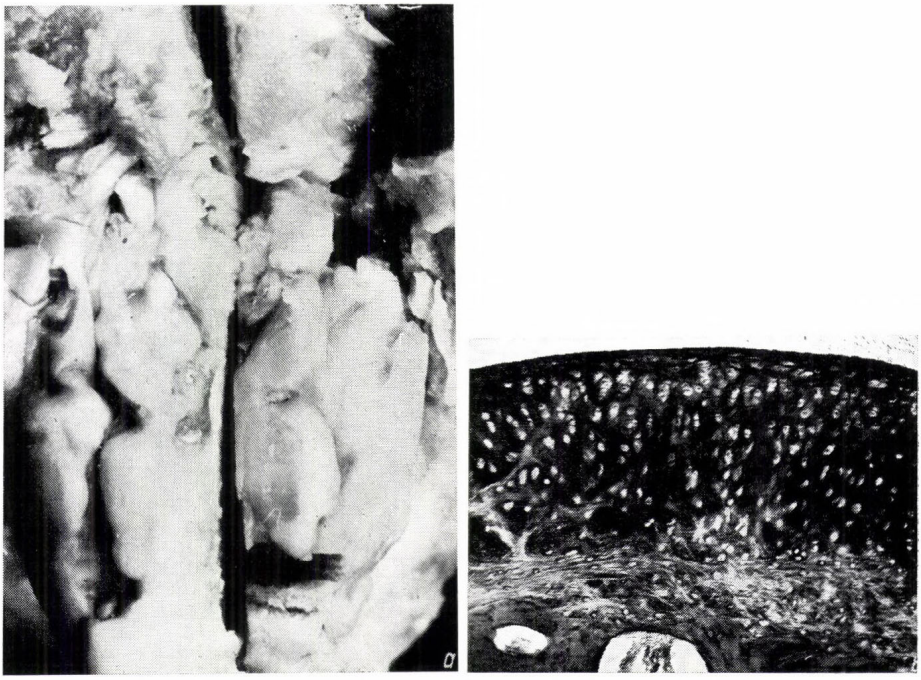

FIG. 5. a) 226 days after operation. b) Microscopic picture of 226-day-old specimen

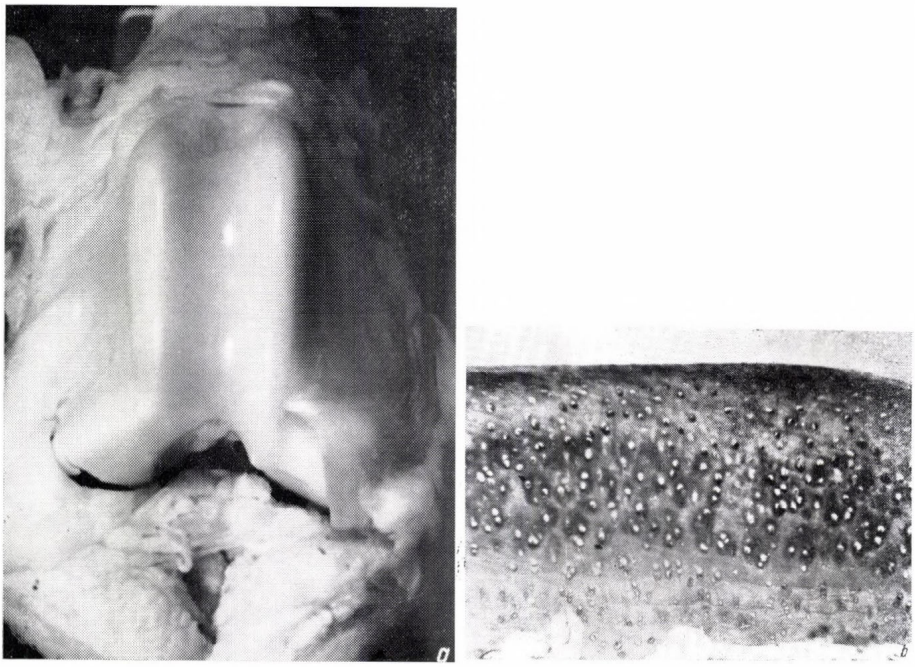

FIG. 6. a) 692 days after operation. b) Microscopic picture of 692-day-old specimen

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BONY UNIFICATION OF VERTEBRAE DUE TO LOCAL ADMINISTRATION OF ADRENAL EXTRACT

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See: *Acta morph. Acad. Sci. hung.* **13,** 25, 1964

Symp. Biol. Hung., 7, pp. 389—390 (1967)

CYTOCHROME OXIDASE CONTENT OF THE ORGANS OF SOME LOWER ANIMALS

by

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The cytochrome activity of different organs of some lower animals living in Hungary has been determined by the method of Pearl et al. (1963) and that of Straus (1954). The results obtained have been compared with the values obtained in the liver and heart of the rat, also known from the literature. In the majority of the tissues of lower animals the cytochrome

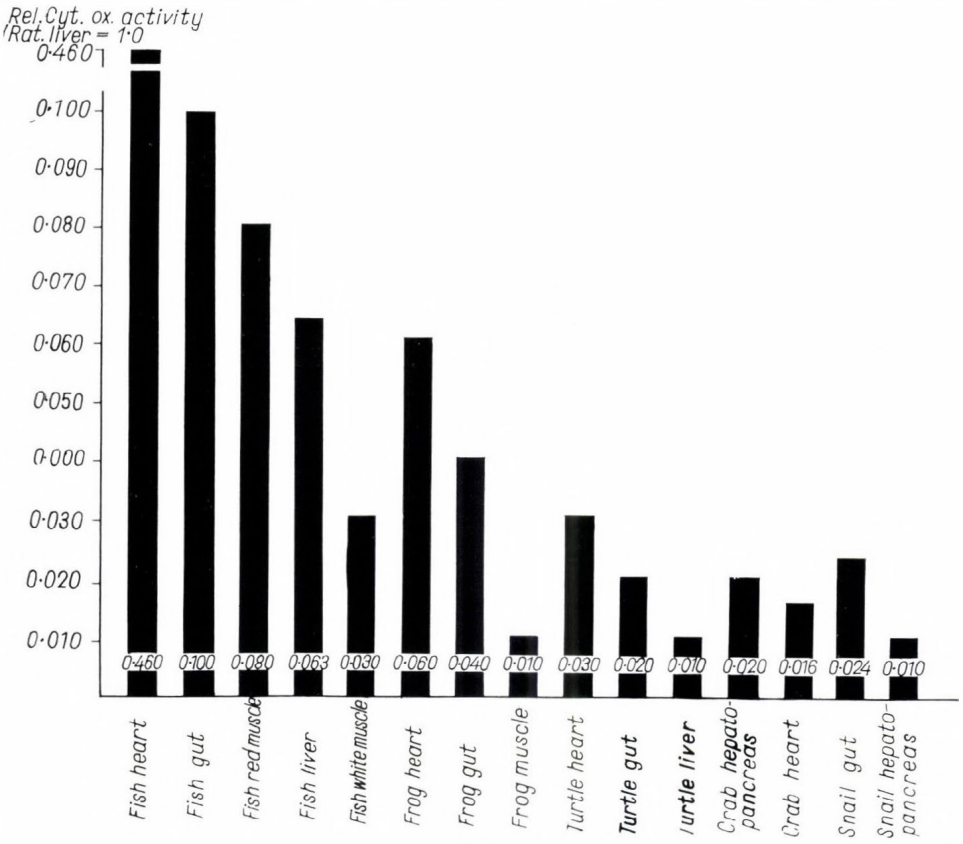

Fig. 1

oxidase activity (see figure) was found to be 50 to 100 times lower than that of the rat tissues. The degree of cytochrome oxidase activity seems to be in connection with the oxygen supply of the tissues, i.e. with their circulatory conditions. Reference is made to the finding that in the tissues of lower animals containing low cytochrome oxidase activity, a high mucopolysaccharide content was demonstrated (Krompecher et al. 1966).

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CYTOCHROME OXIDASE PREPARATIONS

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Symp. Biol. Hung., 7, pp. 391—392 (1967)

EFFECT OF PROLONGED THYROXINE AND METHYLTHIOURACYL ADMINISTRATION ON THE EPIPHYSEAL CARTILAGE OF THE THIRD METACARPUS OF GROWING RATS

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As long ago as in the last century it was recognized that a correlation exists between the dwarfism of cretins and the activity of the thyroid gland. Since the observation of Gudernatsch (1912) an increasing number of authors have investigated the connection between linear growth and thyroid activity (Becks et al. 1942, Silberberg and Silberberg 1954, Hulth and Nylander 1963). From the experimental results it was concluded that thyroxine has an effect on linear growth. The relevant data reported in the literature were, however, often divergent, and sometimes even contradictory.

In the course of our experiments conducted on 72 albino rats, the changes ensuing in the distal epiphyseal cartilage of the third metacarpus were studied over a period of 145 days following daily administration of 10 μg/100 g of thyroxine (Hoffman-La Roche A. G., Basle) and that of 0·05 g of Basethyrin (4 methyl-2-thiouracyl; Chemical Works of Gedeon Richter Ltd. Budapest).

In the thyroxine-treated rats increased cartilage maturation and destruction were observed. At the same time osteogenesis was also moderately increased.

On the distal surface of the epiphyseal cartilage, giant cells appeared in large numbers which are considered by some authors identical with the chondroclasts described by Krompecher (1940; Fig. 1). The epiphyseal cartilage was found to become thinner by the age of 90 days and is closed 5 to 10 days earlier than in the controls.

In the basethyrin-treated rats, in addition to cartilage maturation and destruction, a retarded osteogenesis which exceeded the extent of cartilage breakdown was observed. The epiphyseal cartilage cells were found to be smaller, the zone of maturation narrow, the ground substance relatively poor in cells and the epiphyseal disk was persistent even after 145 days of treatment (at the age of 175 days; Fig. 2). In the controls the epiphyseal closure was found to occur by the age of 115 to 125 days. As regards the

FIG. 1. Persisting epiphyseal cartilage of rat after 145 days of basethyrin treatment (age: 175 days). Staining: Haematoxylin-eosin

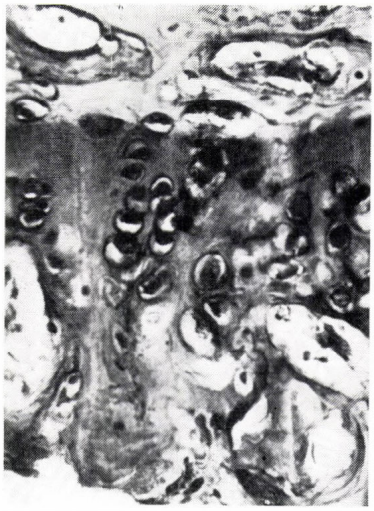

FIG. 2. "Chondroclast giant cell" from the distal surface of the epiphyseal cartilage of thyroxine-treated rat. Staining: Haematoxylin-eosin

mechanism of action, the following questions are raised: either the primary effect of thyroxine or the consequent hyperpituitarism accompanied by the accumulation of acidophilic cells in the adenohypophysis may increase renal Ca and P excretion, (Weinmann and Sicher 1947).

The alterations observed in the basethyrin-treated rats are attributed to hypothyroidism. However, the problem of the peripheral effect of thiouracyl preparations inhibiting oxidative enzyme activity is likewise raised.

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Symp. Biol. Hung., 7, pp. 393—395 (1967)

EFFECT OF LOCAL ADMINISTRATION OF HYALURONIDASE AND ATP ON CALLUS FORMATION

by

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According to the investigations *in vivo* of Paff and Seifter (1950), administration of testicular hyaluronidase results in a retarded longitudinal growth of the femora owing to hydrolysis of chondroitin sulphate. Ludowieg et al. (1961) suggest that testicular and bacterial hyaluronidases split the mucopolysaccharide polymeric chain between glucuronic acid and acetylglucosamine. Adenosine triphosphate (ATP) plays a catalysing role both in the formation of glucuronic acid and in that of acetylglucosamine. Relying upon these findings we have examined the effect of hyaluronidase and ATP on callus formation.

Twenty-four albino rats were used in the experiments. The right femur of each rat was fractured at the middle portion of the diaphysis and the fractured ends were fixed by a small canula used also for the administration of hyase or ATP. One group of rats was treated with 0·15 ml/rat hyase, a second group received, in addition to hyaluronidase, also 0·2 ml ATP/rat in daily doses, through the canula. The third group was left untreated for control. The rats were killed after 10 and 14 days of treatment and the removed calluses were examined by histological, histochemical and statistical methods.

In the 10-day-stage the calluses of the controls consisted of connective tissue containing also some large cartilage islets. In the hyaluronidase-treated rats the calluses consisted predominantly of connective tissue with a minimal amount of cartilage. In the group of hyaluronidase + ATP treatment, the extent of cartilage increased in the callus as compared with that observed after hyase treatment alone.

In the 14-day-stage, in the hyaluronidase-treated group, more numerous cartilage islets appeared than in the previous stage and the first cartilaginous trabeculae were noted. The amount of cartilage was, however, smaller than that of the controls (Figs 1 to 3). In the hyaluronidase-treated rats, the Hale positivity of cartilage cells remained invariable, the basophilia of the ground substance had the same localization as in the former stage, but it decreased considerably in intensity. Its metachromasia was of β-type, while the controls displayed γ-metachromasia. The PAS reaction was similar to that of controls. In the hyaluronidase + ATP-treated rats, the cartilage cells and the ground substance showed an intense basophilia, similar to that of the controls. The statistical data are the results of planimetric examinations (Fig. 4). According to our examinations, local administration of hyaluronidase decreased the formation of cartilaginous callus, owing to depolymerization of acid mucopolysaccharides.

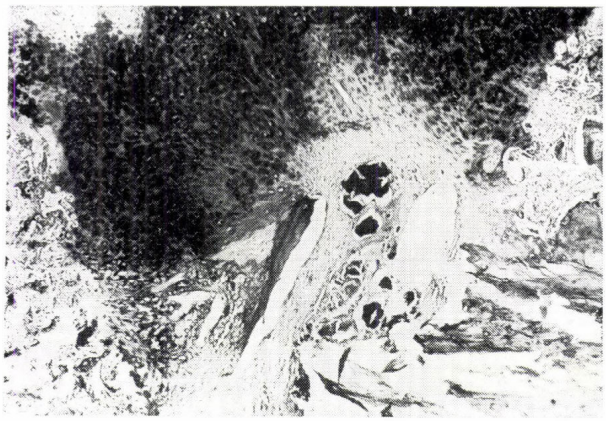

FIG. 1. Site of fracture in the control rat (14-day-stage)

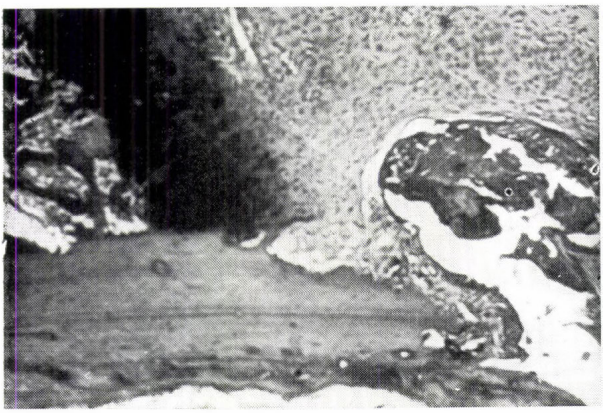

FIG. 2. Site of fracture in hyaluronidase-treated rat (14-day-stage)

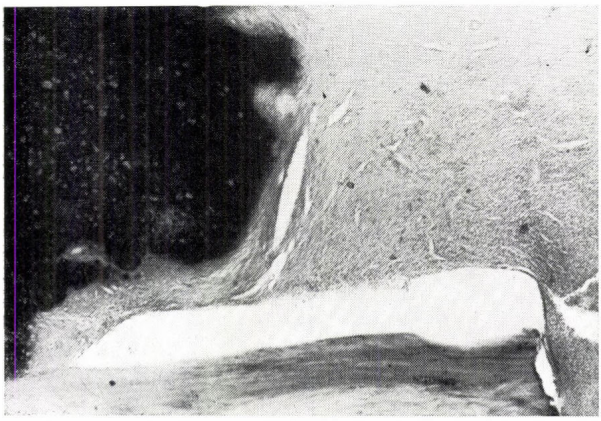

FIG. 3. Site of fracture in hyaluronidase + ATP-treated rat (14-day-stage)

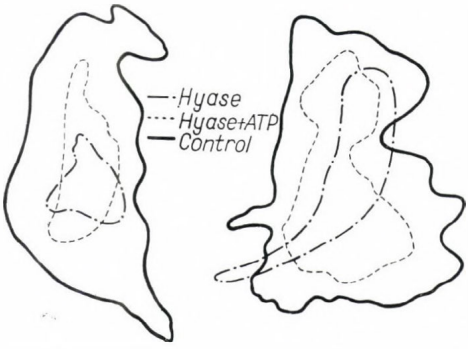

FIG. 4. Contours of superposed largest cartilage islets measured planimetrically. After 10 days (left) and 14 days (right) of treatment

The effect of hyaluronidase was partly compensated by locally given ATP. The formation of acid mucopolysaccharides appeared to be increased by ATP as demonstrated histochemically. These results are in agreement with our former ones on this subject.

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Symp. Biol. Hung., 7, pp. 397—399 (1967)

INVESTIGATIONS ON POSTEMBRYONIC BONE FORMATION IN ALBINO RATS

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Investigations on chondral bone formation have been carried out on the proximal epiphyseal cartilage of the tibia of albino rats from the first day of life until 90 days of age, by means of histochemical methods.

As it is known from the works of Joel et al. (1956), there is a gradual decrease with increasing age in the acid mucopolysaccharide content of the cartilage, and parallel with this process there is an increase in the amount of neutral mucopolysaccharides. The epiphyseal cartilage, considering its essential role in chondral ossification, was supposed to exhibit similar changes in its histochemical pattern.

The methods employed in our investigations were: staining with haematoxylin-eosin and azan for histological examinations; Ritter-Oleson's (PAS + Hale's reactions) technique with acetylation, saponification, supplemented with digestion with pepsin, hyaluronidase and amylase, metachromatic reactions with thionine and toluidine blue at pH 1·4 and pH 3·0; extinction with methylene blue at pH 1·42 and 4·92, reactions with astra violet, astra blue and alcian blue, as well as Wolman's Bi-Col reaction (Földes et al. 1965, Módis et al. 1964). The histological and histochemical findings have been jointly assessed in the light of the ossification stages according to Krompecher (1937).

According to our results, the postembryonic period examined may be divided in three stages.

The *first stage* lasting from 1 to 30 days of age, is characterized by predominant chondrogenesis, the separation of the epiphyseal disk from the articular cartilage and high acid mucopolysaccharide content. In this phase neutral mucopolysaccharides are found only in the bone ground substance (Fig. 1).

In the *second*, intermediate phase, lasting from the age of 30 to 45 days, chondrogenesis and osteogenesis are in equilibrium. Histochemically, neutral protein-sensitive mucopolysaccharide-protein complexes are demonstrated in the distal zone of the epiphyseal cartilage (zone of calcifying cartilage). The remaining zones of the epiphyseal cartilage continue to contain acid mucopolysaccharides. At the end of the intermediate phase (on the 45th day) neutral polysaccharides are demonstrated in the ground substance of the zone of maturing cartilage (Fig. 2).

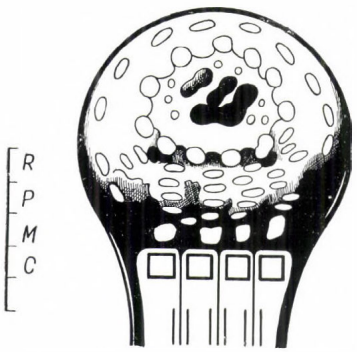

FIG. 1. Epiphyseal cartilage of a 10-day-old rat. The centre of ossification has appeared, the epiphyseal disk is separated

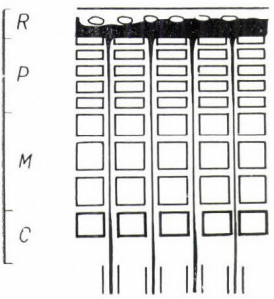

FIG. 2. Epiphyseal cartilage of a 30-day-old rat. Bone and cartilage formations are in state of equilibrium. The epiphyseal cartilage is divided in zones

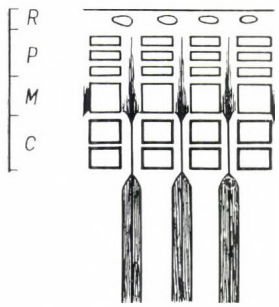

FIG. 3. Epiphyseal cartilage of an 80-day-old rat is narrow. Characteristic predominance of bone formation

The *third stage* lasts from 45 to 90 days of age. Predominant bone formation is characteristic of this stage. The mucopolysaccharides of the epiphyseal cartilage, from the metaphysis, gradually transform into mucopolysaccharide protein complexes. In the last stage (90th day) acid mucopolysaccharides were demonstrated only in the ground substance of the upper layer of the zone of proliferation (Fig. 3).

According to our examinations, the investigation of mucopolysaccharides is adequate for the study of the process of ossification, since the transformation of mucopolysaccharides is parallel to the dynamics of bone formation in ontogenesis.

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Symp. Biol. Hung., 7, pp. 401—404 (1967)

HISTOCHEMICAL INVESTIGATIONS OF CARTILAGE TISSUE

by

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The reactions based on basophilia used for the demonstration of acid mucopolysaccharides are extensively employed in the histochemical differentiation of mucopolysaccharides.

By the term basophilia we mean the propriety of certain tissue components (containing acid groups and high molecular weights) to bind cations (Lindner 1965). In a wider sense, three manifestations of basophilia employed in histochemistry may be distinguished: the phenomenon of metachromasia, the binding reaction of metal colloids and the binding of simple basic dyes. Our investigations have been carried out on the three types of basophilia mentioned above with regard to theoretical and histochemical-technical considerations.

METACHROMASIA

The mechanism of the phenomenon seems to be as follows: the negative anionic groups present on the surface of the tissular macromolecules (mucopolysaccharides, nucleic acids) are bound by electrostatic force to the positive poles of the so-called metachromatic dyes. If the distance between the bound dye molecules is smaller than 5 Å, these molecules are able to polymerize, by which a dye polymerizate is formed on the surface of the polion. The light radiated by this polymerizate differs from the original colour of the dye (Fig. 1).

Grouping the metachromatic dyes according to their β-or γ-metachromasia, the blue dyes proved to be optimal in differentiating these two types of metachromasia (Földes et al. 1964). In all cases the metachromatic reactions should be performed at low pH values. In case of cartilage, Susa fluid should be used for fixation.

REACTIONS WITH METAL COLLOIDS

The binding of iron colloid to mucopolysaccharides is the basis of the reactions according to Hale, Müller, Gömöri, and Rinehart-Abul Haj,

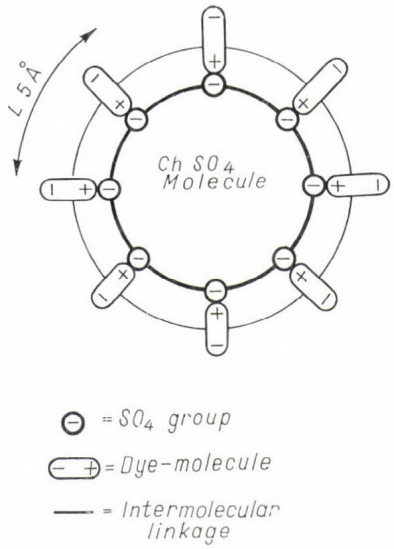

FIG. 1. Scheme of mechanism of metachromatic reactions

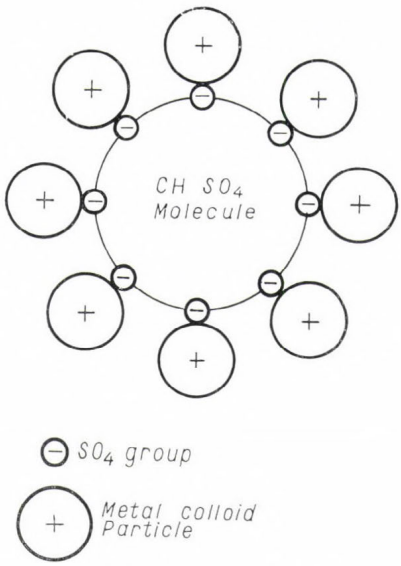

FIG. 2. Scheme of mechanism of colloid metal reaction

as well as the binding of gold colloid in Wolman's Bi-Col reaction. Starting from this statement, we have employed solutions of some heavy metal colloids in carbohydrate histochemistry. Particularly good results have been obtained with molybden, copper and silver colloids at adequate pH values (Módis et al. 1965).

The mechanism of the reaction seems to be the following: large colloid particles having a positive charge are bound electrostatically to tissular anionic groups (SO_4, PO_4). If the colloids are coloured, the effect of binding is visible even without 'developing reagent' (Fig. 2).

BINDING REACTIONS OF NON-METACHROMATIC BASIC DYES

According to our investigations, in addition to basic dyes known from the histochemical literature, some other basic dyes may be employed for staining tissular macromolecules. Some of these dyes are fluorochromes which are particularly suitable for finer cytological examinations. On the basis of the absorption curve, paperchromatographic analysis, chemical structure, physical characteritics and analysis of their colour tone by colour circle, according to Ostwald, we can select the optimal, and technically most reliable basophil reactions. The copper phtalocyanine derivate, Astra violet, was found to be specially suitable for histochemical purposes. The reaction may be examined in light microscopy as well as in fluorescent light (Figs 3—5).

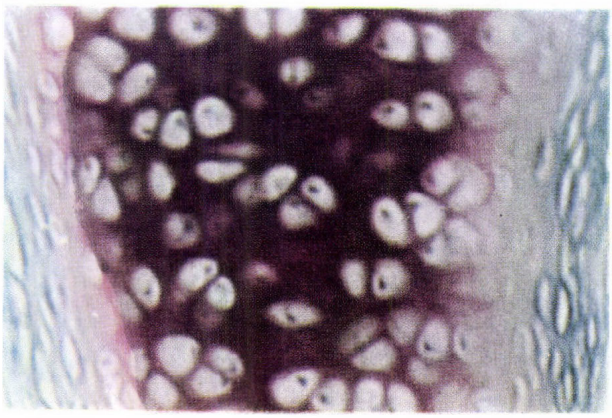

FIG. 3. Astra-violet reaction in laryngeal cartilage of rat. Counterstain: light green

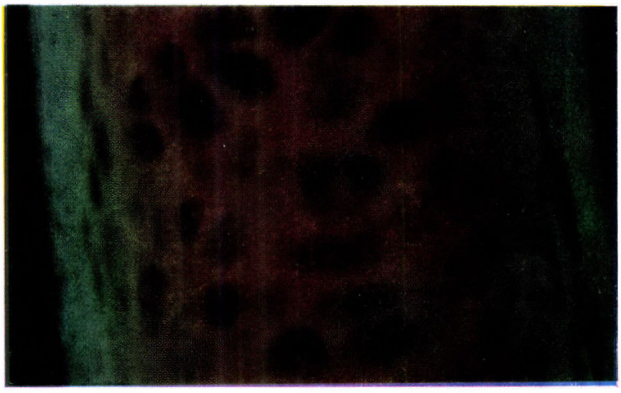

FIG. 4. Astra-violet reaction in laryngeal cartilage in fluorescence microscope (BG 3/4 0G 1/1 filter, Zeiss HBO—50)

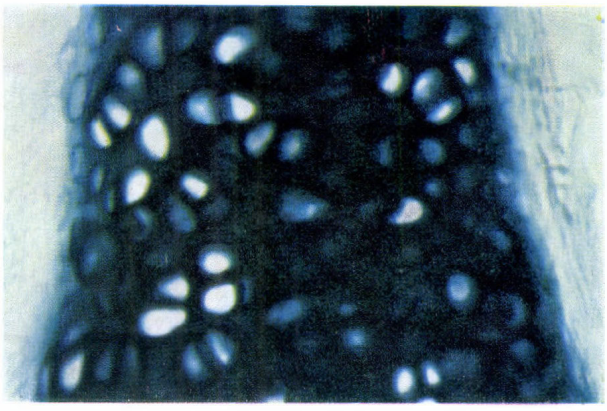

FIG. 5. Control of the former reactions with astra blue

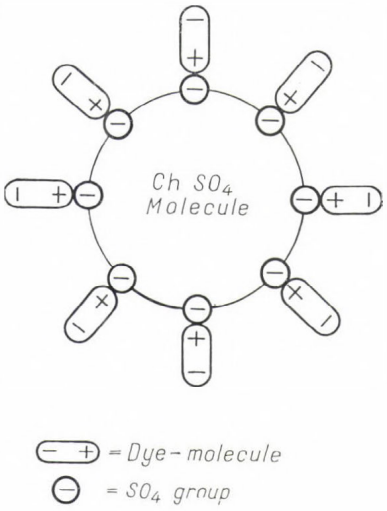

FIG. 6. Scheme of mechanism of reaction given by non-metachromatic basophilic dye

According to the presumed mechanism of the reaction, the tissular anionic groups bind with electrostatic force the 8 positive poles of the dye molecules in solution (Fig. 6).

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HEXOSAMINE PRODUCTION OF RAT-LIVER MINCE UNDER AEROBIC AND ANAEROBIC CONDITIONS

by

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Symp. Biol. Hung., 7, pp. 405—406 (1967)

BIOCHEMICAL STUDIES ON SOME MEDITERRANEAN SEA ANIMALS

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ACCORDING to numerous data in the literature, tissues poorly supplied with oxygen have high mucopolysaccharide contents, but at the same time their lactic acid content is not as high as it would be expected, owing to the anaerobic conditions of such tissues. Similar results were obtained by Krompecher (1960, 1964) Hadházy et al. (1963) on higher animals, and by Gabe (1962) and Krompecher et al. (1966) on lower land-animals, using histological, histochemical and biochemical methods.

Determinations of lactic acid and hexosamine content, as well as cytochrome oxidase and glycolytic activities were performed on some phylogenetically lower sea animals. The determinations on glycolytic activity, according to Warburg's method (Umbreit et al. 1957), and those on cytochrom oxidase activity, measured by the colorimetric method of Pearl et al. (1963) yielded low values. The results obtained from the determinations of lactic acid (according to Barker and Summerson's method 1941) and hexosamine (Boas' method 1953) are given in the Fig. 1, showing a low lactic acid and a high mucopolysaccharide content. The higher the hexosamine content, the lower is its lactic acid content.

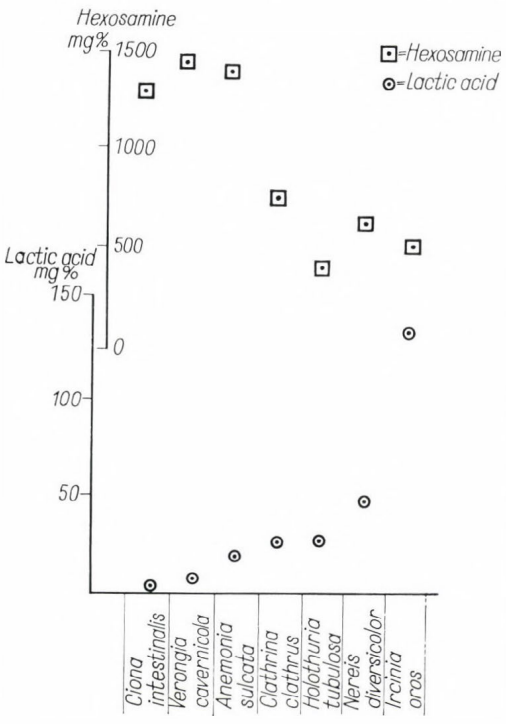

FIG. 1

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Symp. Biol. Hung., 7, pp. 407—409 (1967)

HOMOTRANSPLANTATION OF ARTICULAR CARTILAGE WITH A SHELL OF SUBCHONDRAL BONE

by

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We demonstrated in 1961 (Krompecher and Pap 1961, Pap and Krompecher 1961) that homogeneous transplants of articular cartilage with adjoining cancellous bone survived if they did not exceed 5 mm in thickness and if they were subjected to normal physiological stimuli.

Fig. 1. Homogeneous transplantation of the whole articular surface no thicker than 5 mm; 85-day-stage. Good functional result

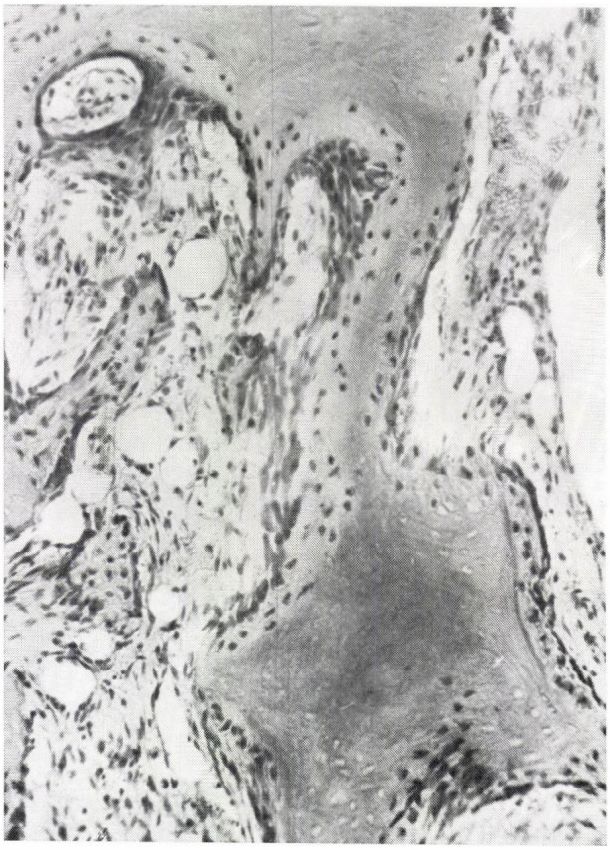

FIG. 2. The homotransplanted bone trabeculae did not survive, but they became surrounded by a living bone layer. The new living bone is easily recognized by the staining of nuclei

The graft was viable, the articular cartilage survived as it could be demonstrated in stages from 8 to 772 days (Figs 1 and 3). The cancellous bone trabeculae did not survive but were gradually resorbed and replaced by new appositional bone surrounding the transplanted bone trabeculae with a layer of living bone (Fig. 2). The transplanted marrow died but was replaced by the host, taking its source from the immigrating granulation tissue and vessels (Fig. 3).

Our experiments in dogs have been repeated and controlled by Campbell et al. (1963) on 42 adult mongrel dogs. The material was studied histologically at intervals ranging from 5 to 500 days. The results obtained by Campbell et al. confirmed our experimental results. Depalma et al. (1963) reported similar findings.

Similar transplantations in human beings have shown encouraging results.

FIG. 3. New bone marrow with new vessels have been built in the homotransplanted bone. See the osteoblastic border. On the top of the picture a segment of the articular cartilage can be seen; 170-day-stage

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HISTOLOGICAL STUDIES ON THE DEVELOPMENT OF HALLUX VALGUS

by

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Symp. Biol. Hung., 7, pp. 411—413 (1967)

STUDIES ON CALLUS FORMATION IN HYPERTHYROIDIC AND HYPOTHYROIDIC RATS

by

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The effect of increased and decreased thyroid activity on growth has been in the centre of many investigations, but there are very few data in the literature concerning the effect of thyroid activity on callus formation. The opinions of the authors are divided. Some researchers suggest that thiouracyl has an inhibiting effect on enchondral and appositional growth (Hulth and Nylander 1963, Silberberg and Silberberg 1954). Thyroxine has been demonstrated as having a stimulating effect on ossification (Kuhn and Hammer 1956, Silberberg and Silberberg 1954, Zimprich 1962).

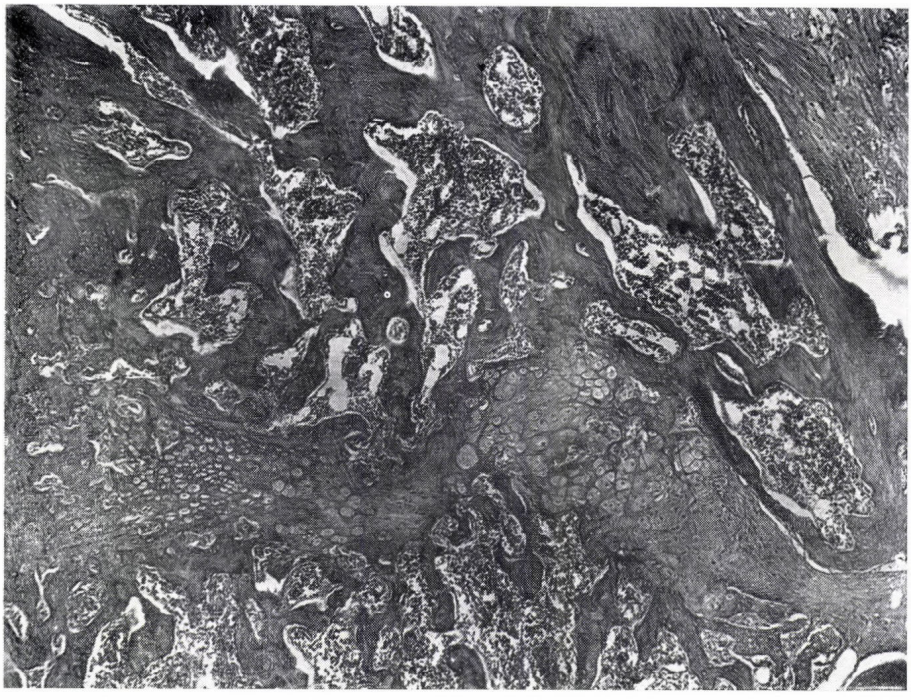

Fig. 1. After 40 days the bone ends are connected by a callus consisting of thick bone trabeculae. In the fracture line there is a thin line of cartilage

FIG. 2. After 40 days, in the controls, the calluses consist mostly of bone tissue, but a wide fibrocartilaginous part is found between the broken bone ends

The purpose of our investigations was to study bone regeneration following administration of substances stimulating and inhibiting thyroid activity.

The experiments were conducted on 45 white rats. One group of rats was given 20 gamma/rat *Thybon* (L 3, 3', 5 triiodthyroninhydrochlorid, Hoechst A.G., Frankfurt am M.). Another group was treated with 30 mg/rat *Metothyrin* (1 methyl-2-mercaptoimidazol, Chemical Works of Gedeon Richter Ltd. Budapest) in daily doses, for a period of 25 days. After this preliminary treatment, the femora of the rats were fractured and fixed by medullary nailing. A group of rats was subjected to bone fracture but otherwise was left untreated. The treatment was continued for 10, 20, 30 and 40 days following fracture of the femora. Then the rats were killed and the calluses and thyroid glands were removed and examined by histological methods.

Twenty days after fracture in the *Thybon-treated* rats, the bone ends were connected mostly by a callus consisting of bone trabeculae. In the *controls* the bone trabeculae of the callus were found to be thinner and smaller in number, cartilage cells and connective tissue being predominant. In the *Metothyrin* group the calluses consisted of connective tissue and cartilage tissue made up of enlarged cells. As regards the extent of the callus, no difference was seen between the groups. However, the greatest qualitative difference between the calluses of the Thybon-treated and Metothyrin-treated rats was noted at this time (20 days).

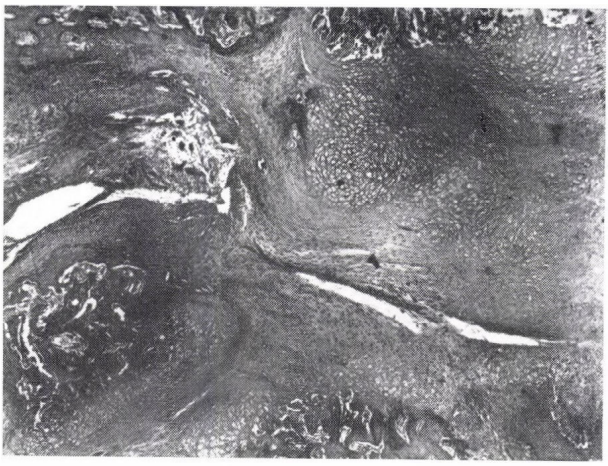

FIG. 3. After 40 days the calluses of the hypothyroidic rats consist chiefly of fibrocatilage with a few bone trabeculae in it

Forty days after fracture the calluses of the *Thybon-treated* rats consisted of thick bone trabeculae with a thin zone of fibrous cartilage in the fracture line (Fig. 1).

In the *controls* the calluses exhibit bone trabeculae and fibrous cartilage corresponding to the fracture line (Fig. 2). In the metothyrin-treated rats the bulk of the large calluses still consisted of extensive fibrous cartilage which was found also between the fractured ends (Fig. 3).

After 40 days there was no marked difference between the calluses of the hyperthyroidic and control rats. In the hypothyroidic rats, however, the bony callus was much retarded.

According to our results, Thybon (hyperthyroidism) exerts a stimulating effect on cartilage resorption and bone formation, two important stages in enchondral ossification (Krompecher 1958). Metothyrin (hypothyroidism) seems to inhibit cartilage resorption and bone building. These effects are in connection with altered metabolic processes and with the increased or retarded vascularization of the callus.

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Symp. Biol. Hung., 7, pp. 415—418 (1967)

THE HEALING PROCESS IN BONE CAVITIES FILLED UP WITH A MIXTURE OF EGG-SHELL AND PLASTER (ANIMAL EXPERIMENTS)

by

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KROMPECHER (1958) was the first to point out the antirachitic effect of pulverized egg-shell as well as the similarities between the chemical composition of bone and that of the egg-shell. Lelkes and Mészáros (1960) reported on a more rapid fracture healing resulting from peroral administration of powdered egg-shell. As a result of these findings, Tarsoly (1963) carried out

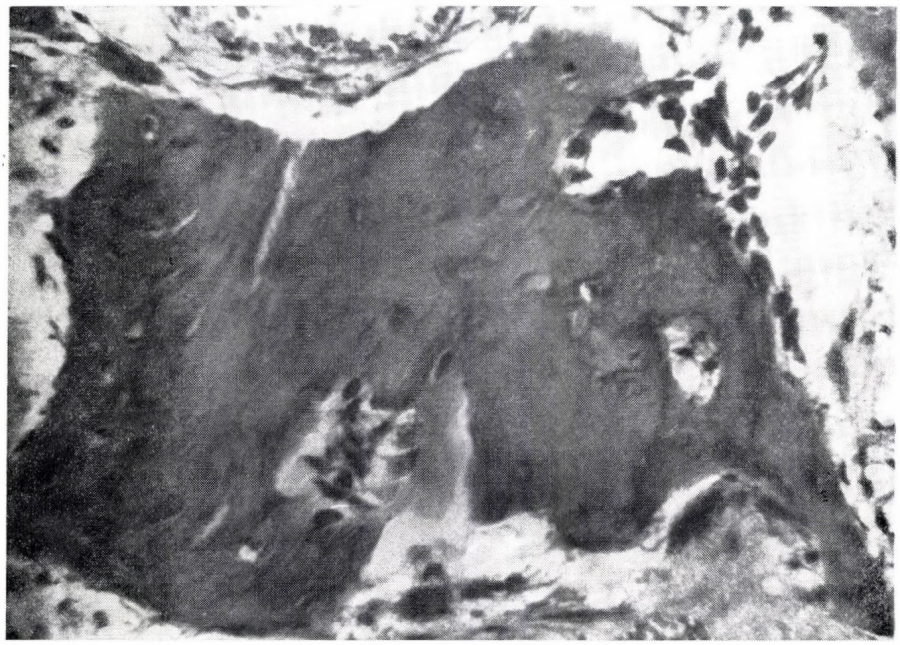

FIG. 1. Ten days after operation: in the cavity filled in with pulverized egg-shell and plaster, osteoblasts are seen on the young trabeculae formed on the surface of the filling material, distributed in islets. The mixture is broken down by osteoclasts

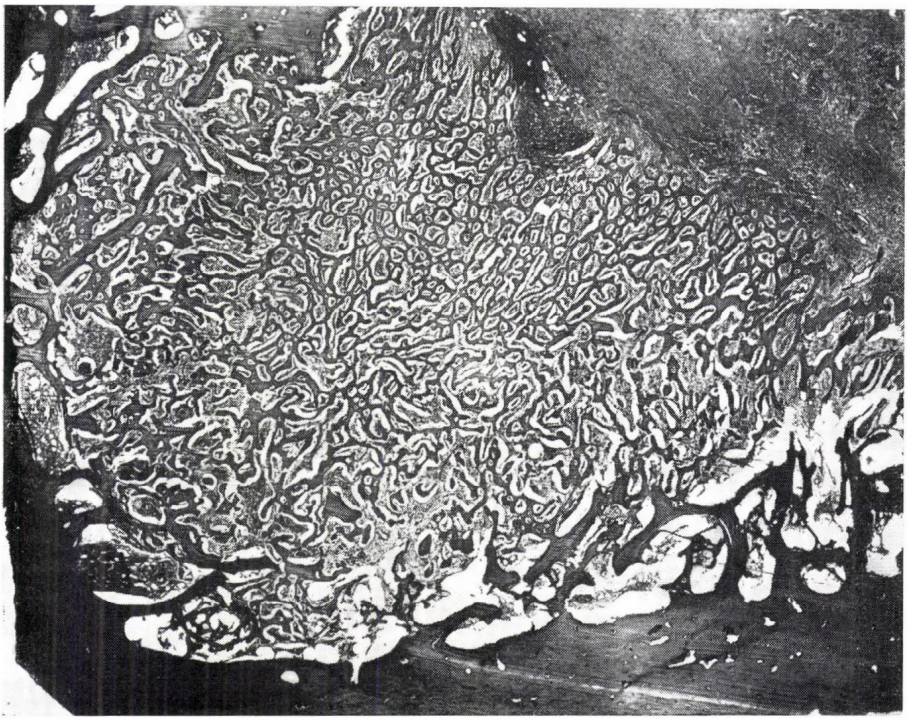

FIG. 2. Fourteen days after operation: a fine network of thin bone trabeculae is observed in the cavity filled in with egg-shell and plaster mixture. The bony callus extends beyond the level of the cortical bone

investigations on the effect of local administration of egg-shell in case of bone defects. In connection with Tarsoly's experiments on dogs, in the course of which holes of 8 to 10 mm dia were bored in the bone and filled in with a mixture of pulverized egg-shell and plaster to stimulate bone regeneration, two problems arose:

1. Whether this procedure can be successfully applied to fill up larger cavities;
2. Whether it may be developed to be applicable in human practice.

Experiments were conducted on 10 dogs. Two cavities with diameters from 10 to 15 mm were chiselled in the proximal end of the tibia. One of the cavities was filled in with a mixture of egg-shell and plaster (1 : 1). Crystalline penicillin and streptomycin were added to the mixture as well as physiological saline to make it more plastic. The second cavity, serving as control, was left empty. The dogs were killed after 7, 10, 14 and 28 days, respectively, following operation. The operative area was excised and subjected to histological procedures.

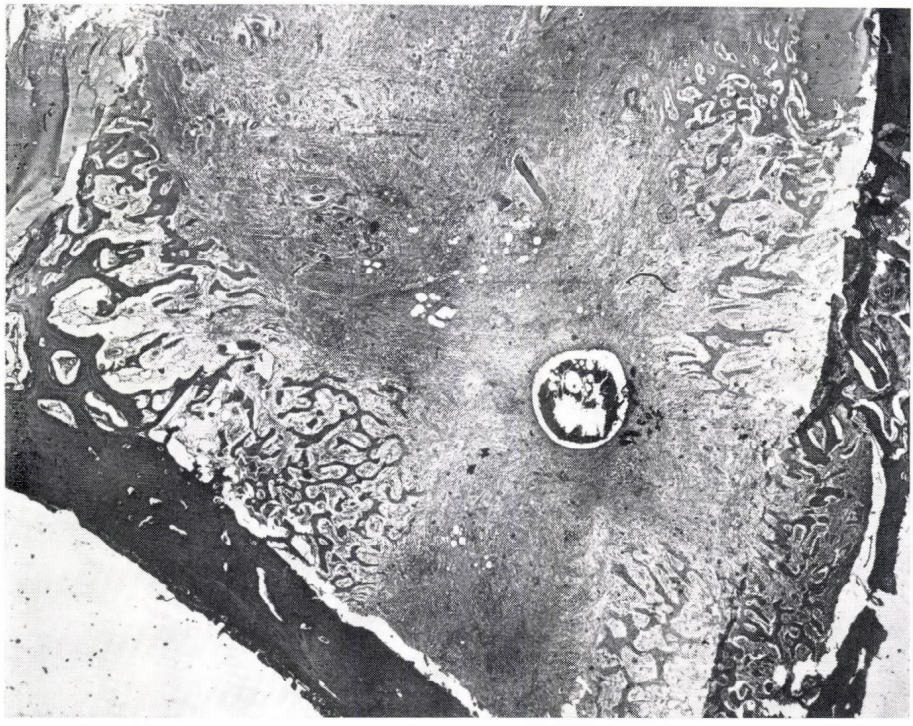

FIG. 3. Fourteen days after operation: the unfilled control cavity contains granulation tissue. A few thin bone trabeculae have formed only on the margins of the cavity

Even seven days after operation, bone regeneration was observed with formation of thin bone trabeculae on the margins of the cavities filled in with egg-shell and plaster mixture. The 7-day-old control cavities contained only connective tissue, no bone formation was visible in them. After ten days the mixture in the inside of the cavity was divided by ingrown vessels and connective tissue, and young bone trabeculae with large numbers of osteoblasts appeared on the surface of the mixture islets, while osteoclasts were active at resorbing the mixture of egg-shell and plaster (Fig. 1). In the 14-day-stage the cavity filled in with egg-shell and plaster mixture was entirely occupied by a fine network of thin bone trabeculae. The mixture was visible only in traces (Fig. 2). In the 14-day old controls, though bone regeneration made its appearance on the peripheral parts, the cavities were still filled up with connective tissue.

Twenty-eight days after operation the cavity was occupied by a marked network of massive bone trabeculae with developed red bone marrow between the trabeculae. In the control cavity, still occupied by connective tissue, new spongious bone was formed only on the margins of the cavity (Fig. 3). Thus, in the cavity left empty, the process of regeneration of bone tissue takes place more slowly than in the cavities filled in with egg-shell

and plaster mixture. Numerous attempts have been made in clinical practice to find out the most suitable material for the filling in of bone defects formed as a result of pathological processes or after surgical intervention necessary in such cases (gypsum, broken bone fragments, polymethyl metacrylate (Bornemisza and Bakó 1958, Borsay and Varró 1959, Fründ 1954, etc.).

According to our experimental results the egg-shell and plaster mixture proved to have a stimulating effect on ossification even in case of larger cavities, besides being an easily procurable and cheap material. Because of its advantages, the mixture of pulverized egg-shell and plaster has been employed in clinical practice with good results (see p. 251).

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Symp. Biol. Hung., 7, pp. 419—420 (1967)

CLOSING ADDRESS

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ALLOW me, as the last day's chairman of this successful Symposium, to speak in the name of all the participants. I wish to express our heartfelt thanks to the Hungarian Academy of Sciences and to you Professor Krompecher and your assistants for the invitation to Debrecen and for the excellent organization of this Symposium.

We have been deeply impressed by the lectures read here and particularly by the achievements of your Institute which we had opportunity to study closer in the demonstration room. At the same time I wish also to thank you for the cordial and friendly reception and hospitality we have experienced here. I ask you Professzor Krompecher to convey our sincere thanks to all the ladies and gentlemen who have taken part in the preparation and organization of this Symposium.

Considering the great number of lectures and subjects, I shall not mention the names of the lecturers in the following summarization of the Symposium.

The starting point of the discussion on callus is the clinical observation. In the course of clinical lectures, chiefly methods of treatment have been discussed which result in the firm connection of the fractured bone ends by regeneration. By direct contact of the broken bone ends the formation of a too extensive callus seems to be avoidable, and a morphologically and esthetically good result can be obtained and, concomitantly, a perfect function. The statement that in each case medical judgment should be preferred to any schematic treatment is remarkable. In any case, every treatment requires abundant experience.

The conclusions drawn from clinical observations and experimental investigations on callus formation and transplantation are very complex. When the mechanism of callus formation has been discussed, it was remarkable that simple mechanical interpretation of the process of fracture healing was mostly declined, though the importance of mechanical factors and that of function were not contested. In assessing the chemical and hormonal influences affecting the process of fracture healing, special importance was attributed to the vascular system in callus formation. The presentation of the phylogenetic relations of supporting tissues have been very impressive.

Another group of investigations was concerned with the histology of callus and bone tissue. They pointed out the structure of the developing callus trabeculae and osteons bridging over, by osteosynthesis, the gap between the fracture ends.

Further lectures dealt with topochemical, autoradiographical and electron microscopical investigations of cell populations. Investigations with tetracyclines are also worth mentioning. In this respect, all lecturers have emphasized that in discussing processes occurring in the intercellular substances, certain tissular and enzymatic occurrences must be investigated. In this connection, different groups of substances as scleroproteins, mucopolysaccharides and minerals have been dealt with. Thus, I have come to the comprehensive presentation of the biochemistry of bone tissue, and in connection with cytological investigations to molecular biology within the frame of callus formation.

Surveying the totality of the subjects discussed here, we have the impression of standing before a lot of fragments just as we have seen in fractures shown in X-ray pictures. But—sticking to the terminology of the Symposium—we have to look for a 'nail' or 'compression plate' which would connect all these subjects in a final unity, just as in the case of a well-healed fracture. Perhaps we can find such a 'nail' if we go back to the older conceptions of Petersen, renewed recently by P. Weiss and W. J. Schmidt, namely to the hierarchy of the scale of orders. By including the facts discussed here in this hierarchy, at one end we find the healthy or sick man and at the other end, the molecule. By penetrating further in the direction of the molecule, we lose Life, according to W. J. Schmidt. The phenomenon of life arises if one advances from a lower degree to a higher one and in the course of this gradual progress each higher step provides more than the simple summation of the degrees below it. This train of thought may be—as a repetition of the theory of totality *(Ganzheitstheorie)* refuted or questioned. Still, according to P. Weiss, we must have the courage to apply it .The healing process disclosed by the X-ray picture can be understood only if we know the histology of the callus, and the latter can be understood only if we deal with the cells of the callus, and the cells are known only if we advance as far as molecular biology. Thus, for the further fruitful investigation of the callus, the barriers between the steps of this scale should be broken through by collaboration of the representatives of different disciplines. The participants of the Symposium have been entirely of the opinion that beyond the stimulus we have received here, we must meet again at a given time. A proposition has been made to form a free association of Callus Symposium which would deal with problems of bone regeneration. As Dr Koskinen has proposed Helsinki for the next Callus Symposium, I think Professor Krompecher and Dr Koskinen should form a kind of Organization Committee to decide upon the place and date of the next Callus Symposium.

Responsible for publication: Gy. Bernát
Director of the Publishing House of the Hungarian Academy of Sciences
and of the Academy Press. Responsible editor: K. Takácsi—Nagy
Technical editor: M. Stelczer
Jacket designed by Gy. Komlosán
Set in 10 pt extended
Printed at the Academy Press, Budapest

Published in the Series

SYMPOSIA BIOLOGICA HUNGARICA

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Distributors: KULTURA

Budapest 62. P.O.B. 149